

## US needs an environmental policy

*Mrs Anne Burford's resignation from the Environmental Protection Agency last week, sad but not tragic, is a reminder that the Reagan Administration is bereft of what might be called a policy.*

THE departure last week of Mrs Anne Burford from her post as administrator of the Environmental Protection Agency — and the manner of it — could have happened only in the United States. Although the head of an agency may be appointed by the administration (technically, by the President), there are many circumstances in which an agency's responsibility for the administration of legislation is so onerous that its head is for practical purposes responsible to Congress. But the knowledge that last week's affair is another illustration of an admirable feature of the United States constitution is unlikely to be much comfort to Mrs Burford, obviously bruised by her experience.

Her troubles with Congress began last year, when she followed President Reagan's instructions not to make agency documents available to Congress, and earned a legal suit for contempt of Congress for her pains. By the end of last week, it had become plain that, in spite of earlier statements to the contrary, the Reagan Administration would not stomach the fight necessary to help her keep her job. The outcome is unfortunate not merely for Mrs Burford personally, but because her departure will be counted as the consequence of yet another squabble between Congress and the White House. The administration has for the time being ducked the most urgent need — to resolve the issues on which it is at odds with Congress on environmental policy.

President Reagan swept to power in 1980 after an election campaign in which he promised to liberate US industry from regulations, especially those spawned in earlier legislation on the environment. Up to a point, the President has kept his word. The Council on Environmental Quality has all but disappeared. He has sought to make the Clean Air Act less onerous, but Congress has not responded. And the Environmental Protection Agency under Mrs Burford has legally taken advantage of the flexibility within the law to reduce the burden of environmental regulation.

The snag is that the administration has in most of these matters preferred to work by stealth, using what leeway it can find in the legislation wished on it by its predecessors. It has thus won the worst of all worlds: environmentalists suspect the worst (and the Secretary of the Interior, Mr James Watt, gives them ample and frequent justification), Congress has the impression that its wishes are being ignored and yet the administration is without a coherent policy on environmental issues.

Instead of a policy, the administration has only a loosely articulated set of prejudices, one of which seems to be that the United States would be happier and more prosperous if only environmental legislation could be dispensed with. But that will not happen. Congress would not allow it, while even recent experience, the trouble with dioxin in the Mississippi Valley for example, shows that a bonfire of legislation could be disastrous. The only respect in which the administration's prejudices can be widely shared is in its suspicion of a body of legislation with roots in the great and frequently misguided environmental movement of the 1960s and early 1980s. How, the argument seems to go, can laws drafted in that heady atmosphere be appropriate now.

That suspicion is misplaced. There might be something in the view that Congress would not have acted on clean air and clean water legislation if there had not been an army of self-styled ecologists prepared to pack the space around the Washington Monument whenever a new environmental insult came along, or some new species appeared to be threatened, but the need for

legislation of some kind is plain. The later legislation on toxic chemicals is similarly necessary, while the excesses of the earlier drafts have long since been modified. The common and enduring fault in the legislation Congress has wished on the administration lies not in the standards that have been set (and which could presumably always be changed) but in the way in which the federal and state governments have been saddled with responsibility for licensing a host of activities they do not properly understand. One result is a plague of lawyers, constitutionally inescapable.

So what can the administration do? More than a decade after the environmental movement peaked, and with the experience that has since accumulated, it would be entirely proper to embark on a thorough review of the standards set in the past, making intelligent use of information now accumulated of the costs entailed by particular measures. It would not be surprising if one result of such an inquiry were that the use of DDT and other organochlorine pesticides, now almost entirely banned, were found in suitably defined circumstances to be permissible again. In other respects, tighter standards would no doubt be found appropriate, to reduce the acid rain on Canada for example. One benefit of such a review of US environmental policies would be to prompt a switch from the present tendency to control environmental pollution by means of emission standards applicable to all to a system in which more explicit allowance is made for the capacity of the natural environment to absorb a measure of pollution without harm. (Should auto-emission standards in, say, Oregon be identical with those applied in Chicago or New York City?) There would also be great advantages if the enforcement of environmental legislation were to rely less on supervision by federal and state agencies and more on the provision of effective penalties for offenders.

It is a great surprise that the administration has not thought of following such a tack. Its credentials, and its declared scepticism of the value of much of the environmental legislation would have given it a licence to do just that. The danger that such an inquiry would have provoked a supposedly dormant fury of environmental extremism is no longer real, for times have changed. It is even possible that an intelligent review of environmental policies would persuade Congress that many present laws and practices on environmental matters need to be changed. So why does the administration rely instead on empiricism? Indolence rather than cowardice seems to be the explanation, but even now it is not too late. Surely even President Reagan will recognize that he owes Mrs Burford no less. □

## What cure for Britain?

*A House of Lords Committee has said sensible things about industrial research and development.*

SPECULATION about the causes of the British disease, the persistently poor economic performance of the country in which began the industrial revolution of the nineteenth century, has been rife at least since the end of the Second World War, but has usually been unproductive. The obvious difficulty is that the aetiology of the condition is complicated. Some parts of the causation of the disease are economic — the persistently low rate of investment in manufacturing industry stretching back over decades for



example. Most, however, have to do with people's behaviour. The poor state of industrial relations (of which strikes are only the most spectacular signs) reflects the continuation of the old class structure of British society in the division between managers and those who work for them — as does the persisting class distinction between academic and industrial scientists. The civility of British society is also, in its way, a discouragement of entrepreneurship — nicely illustrated by last week's account of how it has fallen to government agencies to organize (not yet successfully) a biotechnology company functioning in the agricultural field. And by now nobody can dispute that the educational system, based on that devised more than a century ago for the education of a tiny proportion of the British population, produces ill-educated specialists is more than merely an obstacle to prosperity. (Obstacles, after all, can be surmounted.) So what can be done to cure the sickness?

## Advice

The Select Committee on Science and Technology of the British House of Lords has now joined the throng of those proffering advice on what should be done, but to good effect. After an inquiry into the condition of research and development in British engineering industries (HMSO, £6.10), the committee has put forward a string of practical recommendations which, it is to be hoped, somebody (i.e. the British Government) will take seriously. Obviously the proposals cannot constitute a full cure, but they are almost certainly necessary ingredients. Like others embarking on the understanding of some aspects of the British disease, the House of Lords committee has been in part frustrated by circumstances that are themselves part of the trouble. Thus it turns out that the latest figures for spending by British companies on research and development relate to 1978 (and, by all accounts, are shot through with the unreliability that stems from imprecise definition). Properly, however, it is impressed with the way in which British patents registered in the United States have been a declining share of all foreign patent registrations in the past twenty years.

Perhaps inevitably, the committee starts from the assumption that the government must continue up to its neck in the support of industrial research and development. Part of the difficulty in Britain is that several government departments are separately and differently involved. The Ministry of Defence, the biggest spender, is also extreme in the way in which it concentrates on the development of new weapons systems without much caring whether the consequences of its spending will help to improve industrial performance. At the other extreme is the Department of Education and Science, from which flows the money ultimately spent on research in academic institutions. The Department of Industry comes in between — and has won modest praise from the House of Lords for its scheme now renamed "support for innovation" by means of which research and development projects by industrial companies can qualify for partial support with public funds. The pattern of public spending on research and development is as lopsided as ever. Of government support for research and development ending up in industrial companies, the committee estimates that no less than 81 per cent is for defence, while civil aerospace takes a further 12 per cent. Public support for general engineering, on the other hand, amounts to merely 4 per cent of British public money finding its way into industry's research and development laboratories. The committee could with advantage have been more explicit in its complaints about this distorted pattern.

The central conclusion of the House of Lords committee is more polite — that the British Government needs to hammer out a coordinated policy of support for selected areas of industrial research and development, and that a greater proportion of what it spends should go to general engineering. The trouble with co-ordination is that everybody pays lip-service to the ideal but that British government departments are as unwilling as the next fellow to see their autonomy compromised even when the government collectively is persuaded that something must be done. The need, however, is urgent, partly so that industry can

know where it stands but also to moderate the enthusiasm for pet projects particular departments. It has always been and remains a mystery that the British Government sets so much store by civil aerospace, for example, especially now that the principal company in the field has been denationalized and because its business (which includes satellite construction) is profitable. Similarly, there is a serious possibility that the government may have gone over the top in its recent enthusiasm for what is called information technology; the field is obviously important, but the need to spend public money in one of the few fields in which private investment flourishes is far from self-evident.

So how is a strategy to be worked out? The obvious gap in what the House of Lords proposes is that its proposals so manifestly fall short of what is needed. Knowing what happened to its suggestions in last year's report on the organization of technical advice that existing committees should be strengthened and new committees formed, the House of Lords has shrunk from specifying some new coordination mechanism. Instead, it advocates the use of the National Economic Development Office (for which the present government has little enthusiasm) assisted by other bodies such as the Advisory Council for Applied Research and Development and even the Fellowship of Engineering, a kind of national academy for engineers. Unfortunately, these devices will not wash. The advisory council (not for want of trying) has made very little headway in its attempts to inquire into the activities of the Ministry of Defence. The National Economic Development Office, a tripartite entity on which government, industry and trades union are somewhat formally represented, has little capacity for sustained intellectual work. The House of Lords would have been better advised to throw back at the government the response it got when asking last year for a mechanism for coordinating advice — that the Prime Minister, Mrs Margaret Thatcher, herself a scientist, would be ultimately responsible. Will she now rise to the challenge the House of Lords has thrown down, and take on even the Ministry of Defence? And does she have the time?

The committee's most interesting proposals are concerned with commercial questions usually neglected in broodings on the British disease. Stoutly, it says that there is no prospect of self-sustaining industrial research and development so long as British industry is not truly profitable, which is correct. Government handouts are thus but palliative. Soundly, the committee rejects the advice that spending on research and development should qualify for tax relief over and above the present arrangements, which allow a company to deduct the full cost from its operating expenses even though research and development is strictly speaking a form of capital investment (which is ordinarily met from taxed profits). But the committee does advocate a measure of tax relief for people and institutions investing in research and development, a proposal for which there are precedents and which could powerfully stimulate private investment in innovation. This is a tangible proposal that should be urgently explored.

## Dissent

For the rest, the committee's report is a curious mixture of good and less good. Bumbledom prevents the Department of Industry from making grants (up to a third of total cost) when a project has already begun, when a company cannot prove that it would not carry out a project on its own account or when a grant has already been made for a similar project; this should be changed. Government research establishments (which between them cost £900 million a year) are indeed, as the committee says, too separate from industry (and probably too large). Government departments in general, but especially the Ministry of Defence, should indeed contract out more of their research. But it is doubtful whether there is any merit in the proposal that six British universities should be singled out as places where industrial research would ordinarily be carried out. If such activities are valuable (as they are), why should not all universities decide for themselves what degrees of industrial research would suit their special circumstances?



## Biotechnology patents

# New move confuses US patents world

Washington

PATENT attorneys are complaining of "total chaos" over what is required in filing a genetic engineering patent in the wake of a surprise decision by the US Patent and Trademark Office to drop its earlier objections to the second Cohen-Boyer patent application, which covers basic processes in genetic engineering.

The patent office's decision, which was transmitted to Stanford University (which, along with the University of California, holds rights to the patent) on 21 January, was not announced, in keeping with Stanford's request last December to close public access to the patent proceedings. Kathy Ku of Stanford's Office of Technology Licensing confirmed, however, that the patent office had dropped its two principal earlier objections, while raising one new objection. She refused to elaborate.

The confusion centres on what an application must do to meet the patent law's disclosure requirement that a patent must provide enough detail to allow a person with "ordinary skill in the art" to duplicate the invention. Fred Whisenhunt of the Washington firm Murray & Whisenhunt explains that "The bar in general has concluded from the practice of the patent office that when you have a new organism, the only way to have a complete disclosure is to deposit that organism in a public depository".

Depositories such as the American Type Culture Collection are commonly used for this purpose. The patent office's decision in the Cohen-Boyer case, however, has cast a cloud of doubt over this assumption. "According to a lot of members of the bar," Whisenhunt said, "it is a substantial departure from past practice and leaves unanswered what is acceptable."

"The bar is confused", said another attorney familiar with the case. "It's not fair that some people are playing it strictly by the rules and other people are getting away with murder. What is needed are some guidelines — and promptly."

The unusual facts of the Cohen-Boyer case have brought the disclosure issue into sharp focus. The patent application covers the basic process for inserting foreign DNA into bacterial plasmids. Trouble for the patent began last summer, when the patent office became aware that the recipe set out in the application for making pSC101 — the plasmid used to carry the foreign DNA — was incorrect; this raised the possibility that a person with "ordinary skill" would not, in fact, be able to reproduce Cohen's and Boyer's work by following the disclosure in the patent.

But Stanford had a fall-back argument.

The plasmid, they said, was made readily available by Cohen to anyone who requested it, subject only to some safety guidelines and a request that it should not be passed on to others. Thus the plasmid was publicly available at the time the application was filed — and the application need not therefore disclose how to make it.

By dropping its objections, the patent office apparently accepted this claim — much to the surprise and consternation of many patent attorneys, who argue that a private collection maintained by the inventors is no substitute for a public depository. "Based upon what we know, we really think the patent office made a mistake", said Barry Bretschneider of Wegner & Bretschneider, although he emphasized that the full implications of the patent office's action will not be known until the details of the decision are made public.

Whisenhunt says that there are no assurances that a specimen in a private collection will continue to be available to the public. "Another concern is that when you deposit in a public depository you can always determine the characteristics of the organism at the date of filing" — while a sample in a private collection may be replaced with subsequent versions over the years, thus obscuring the true record of what the patent actually covers. "There are members of the bar who feel this is fraught with dangers", he said.

While confusion persists, there is no agreement about the best way of reforming the patent office's procedures. Bretschneider, for one, is doubtful that the problem could be solved by the patent office drawing up strict guidelines, as has been done in Japan. "I'm not confident that our patent office has the experience to issue guidelines that would be applicable in all cases, and I think they understand that."

Meanwhile, the Cohen-Boyer patent may be headed for more trouble. In addition to the new objection raised by the patent office, evidence has come to light of a prior publication that may bring into question the novelty and originality of Cohen's and Boyer's work as defined by the patent law. The publication was a PhD thesis by Stanford graduate student, Peter Lobban, which sets out the complete conceptual framework of the recombinant DNA process.

Although Cohen was the first to do the actual laboratory experiments, the thesis — dated May 1972, more than a year before the Cohen and Boyer publication — could defeat the patent application if it could be held to have made the subsequent work "obvious". The American Patent Law Association's Chemical Practice Committee has a programme of collecting publications relating to genetic engineering and supplying these to the patent office; the Lobban thesis was forwarded to the patent office by this group on 2 March. Several patent attorneys who represent licensees or potential licensees of the Cohen-Boyer patent are thought to have been dismayed at this development on the grounds that they have been hoping to keep the Lobban thesis as ammunition for litigation against the patent after it had been issued.

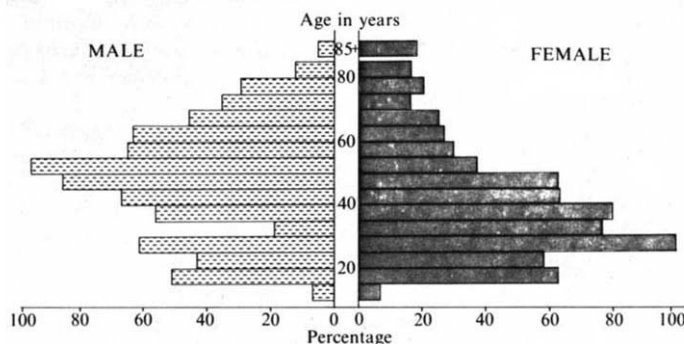
Stephen Budiansky

## Cancer incidence in England and Wales

TAKEN from the recently-published 1978 cancer statistics for England and Wales, the figure shows the percentage increase in incidence of cancer of each five-year age group over the previous one, excluding the age groups 0 to 10, which would mask the general trends since there is an uncharacteristically high incidence of cancer in the youngest age group. Both males and females show a sudden increase in the number of registrations of cancer, but there is a sex difference in the age at which this occurs, being for females between 25

and 30 and for males between 55 and 60. At this point the number of males registering cancer rises to almost double the number of females of the same age, a phenomenon which corresponds with, but is not explained by, a dramatic rise in the incidence of cancer of the lungs and respiratory tract in males over 50. There is no comparative single cancer site which explains the sudden rise in the incidence of cancer in women. Source: *Cancer statistics; registrations* (HMSO: London, £8).

Melanie Kee





## Chemical weapons treaty

# Super-toxics listed by Britain

THE British Government last week tried to break the deadlock in the negotiation of a treaty to ban chemical weapons by urging on the Committee on Disarmament in Geneva the feasibility of inspection of chemical plants to verify compliance with a treaty. But the chances of rapid progress appear slim. The working group set up by the committee to work on the chemical weapons treaty has been hamstrung since the beginning of February by Soviet objections that the chairman should not be a Canadian.

The British proposals on verification are designed to take advantage of the Soviet agreement last year that on-site inspection should be a part of a chemical weapons treaty. They are based on a survey carried out in the British chemical industry of the production for civil purposes of chemicals that could be used for making lethal poisons. The paper presented to the working group in Geneva concludes that inspection to ensure that industrial chemicals are not diverted to military purposes should not impose "undue strain on industry".

The intended treaty would require member states to declare and then to destroy stocks of chemical weapons and manufacturing facilities, which would require once and for all verification. The British paper now argues that members of the treaty, who would also have to declare which manufacturing plants make chemicals that could be used for making weapons, should agree that these should be subject to random inspection. This would be done by teams of specialists working for a permanent organization set up by the treaty. The thorny question of the frequency of inspections remains to be decided.

The British paper emphasizes the need that inspections should be strictly random, and should extend to stockpiles of chemicals as well as production plants. The experience of the safeguards inspectorate of the International Atomic Energy Agency is commended as an example. The proposed inspectorate, the paper says, should be free to develop its own methods within broad guidelines that would be a part of the

final treaty.

Two classes of chemicals are distinguished — those such as hydrogen cyanide and phosgene that are produced in large quantities but of necessity quickly used, and those that are potentially precursors of "super-toxic lethal chemicals". The British Government said last week that these materials are ordinarily produced only in small quantities. None of those on

## Yellow rain

# Not poison but pollen?

Washington

An Australian analysis of "yellow rain" samples on leaves obtained in Thailand has concluded that the yellow spots were "faked and deliberately applied by a brush or spraying device" and that they consisted primarily of pollen from a rain-forest tree. The analysis, performed by Australian government scientists at the Materials Research Laboratory (MRL), failed to detect any toxins in the samples, but did identify the presence of a *Fusarium* mould, apparently growing on wet packaging that the samples had been placed in.

The US State Department has maintained that yellow rain is a toxin weapon, produced and used under Soviet supervision, that contains trichothecene mycotoxins,

its provisional list (see table) is manufactured by more than two British companies, and some are not made at all.

In practice, at least in the Western chemical industry, the declaration by manufacturers of the chemicals they manufacture would not be unduly burdensome, given that such information is now required under occupational safety legislation. By all accounts, last week's initiative by the British Government has the approval of the United States as well as of other European governments, the French included. □

which are produced by *Fusarium* fungi.

The new Australian findings appear in an unpublished MRL report, *Examination of Yellow Rain Specimens Received at MRL April 1982*. Although the report is unclassified, it is marked "not for distribution to members of the press", and has not been made generally available. A knowledge of its contents has, however, been gleaned from a former American Friends Service Committee fieldworker, Jacqui Chagnon, who was allowed to read the report at the Australian Embassy in Bangkok and take notes, and from an American scientist who has a copy of the report.

The samples analysed were from a border region of Thailand that had allegedly been the site of a yellow-rain attack from Kampuchea in February 1982. A Canadian medical team in the area examined villagers and reported finding symptoms such as vomiting and abdominal pains. Laboratories in Thailand, Canada, Australia, Britain, France and the United States all obtained samples of yellow spots that appeared on the vegetation around the time of the alleged attack. As reported by the State Department in November, both US and Thai scientists detected mycotoxins in these samples, which consisted of an unidentified "yellow powder".

In the September issue of an obscure Thai medical journal (*Siriraj Hospital Gazette* 34, pp 643-647), a report on microscopic examination of the samples appears; the powder was found to be largely pollen and, in 22 of the specimens, the fungus *Fusarium tricinatum*, which produces the mycotoxin T-2. The State Department now says that it, too, has found pollen in many of its samples. But, said Gary Crocker of the State Department, "It's not wind-blown pollen, it's commercially-collected pollen". The State Department's theory is now that pollen is added to the yellow rain mixture to provide a delivery vehicle for the toxin. Crocker says that all the pollen is in the 10-20  $\mu$ m range, "the perfect size to be retained in the body". Crocker said that pollen analyses at the Smithsonian Institution identified the pollen as daisy, chrysanthemum, and

## Adding to confusion

Canberra

THE Australian investigation was undertaken at the Materials Research Laboratory of the Department of Defence Support by Dr Hugh Crone. Although the report of the investigation was complete last August, and has been circulating within the defence establishment, no decision has been made on its release to the public.

Mr Anthony Street, then Minister of Foreign Affairs, told the House of Representatives on 18 August last year that the sample of vegetable matter analysed at the laboratory had been gathered in Laos and taken to the refugee camp at Ban Vinai in Thailand. From there, the sample was collected by an official of the Australian Embassy in Bangkok and transferred to the Materials Research Laboratory.

Australia is a co-sponsor of the resolution that established the United Nations committee of experts now examining US allegations that chemical weapons are being used in South-East Asia and Afghanistan, and has agreed to investigate reports of the use of such weapons. The results of this examination, which will be conveyed to the UN committee, add to the confusion surrounding the yellow rain controversy.

Vimala Sarma

## British list of key precursors for super-toxic chemicals

Phosphorus trichloride (PCl<sub>3</sub>)

Phosphorus oxychloride (POCl<sub>3</sub>)

Chemicals containing the *P*-methyl and/or *P*-methyl and/or *P*-ethyl bonds

Methyl and/or ethyl esters of phosphorous acid

3,3-Dimethyl butanol-2 (pinacolyl alcohol)

*N,N*-disubstituted  $\beta$ -aminoethanol

*N,N*-disubstituted  $\beta$ -aminoethane thiol

*N,N*-disubstituted  $\beta$ -aminoethyl halides (halide = Cl, Br or I)

Phenyl, alkyl or cyloalkyl substituted glycolic acid

3- or 4-hydroxypiperidine and their derivatives

several other species.

But Dr Joan Nowicke of the Smithsonian's Natural History Museum, who was consulted by scientists from the Army's Chemical Systems Laboratory (which has done much of the yellow rain analyses for the State Department), said she did not actually perform any analyses for them. She said three people from the Army laboratory came for a one-day crash course in pollen analysis and "then a couple of times I had calls from people who wanted to become palynologist over the phone." She added, "I don't see how they could say it's commercially-collected," since widely varying species have similar pollen that is difficult if not impossible to distinguish.



Crocker of the State Department argues that the Australians' conclusions do not detract from the large body of evidence that suggests use of toxin weapons in South-East Asia. "What is really bothering me is that this one weird example is being used to make a supposition that all samples are fakes", he said. And, referring to the first press report on the Australian analyses, which appeared in the London *Observer* of 6 March, Crocker said, "I tend to think that the *Observer* piece is a fairly deliberate plant to discredit our work."

Professor Matthew Meselson of Harvard University, a critic of the State Department's views on yellow rain, agreed that it was unlikely that all yellow rain samples were deliberate fakes. But he offered a third explanation. Meselson claims that if all the samples are indeed made up of pollen, it opens up the possibility that it's a natural phenomenon. If the pollen turns out to be from local species, and matches the seasonal variation in species, Meselson said, then it is "extraordinarily improbable" that yellow rain is a weapon. Meselson suggests that the known habit of *Fusarium* fungi feeding on pollen could explain the presence of mycotoxins in conjunction with the pollen spots.

**Stephen Budiansky**

## US defence research

# No pot of gold for universities

*Washington*

ALTHOUGH many US university scientists believe that the defence budget is their new cornucopia, they have an unpleasant surprise in store. The huge increases in defence research and development funds that have characterized the two budgets proposed by the Reagan Administration do not show corresponding increases in defence basic research. While the newest budget for fiscal year 1984 shows an 18 per cent increase for the National Science Foundation for example, overall increases in defence basic research will rise by only 9 per cent to \$850 million — an increase of only 4 per cent after inflation.

In current dollars, obligations for defence research and development have been skyrocketing relative to those for all other agencies. When the 1984 budget proposals are taken into account, defence research and development would rise further, to \$24,000 million, an 18 per cent increase over fiscal year 1983. Meanwhile the research and development budgets for all other federal agencies would remain at about \$18,000 million. The money requested for college and university basic science would go up by only 1 per cent, from \$425 million in 1983 to \$434 million in 1984.

A detailed analysis of mathematics and computer science funding in the proposed 1984 budget, recently prepared for the American Association for the Advancement of Science, shows how defence basic science funding lags behind that for civilian agencies. Although most of the military funding offices show some increases, there are falls in the Office of Naval Research's budget for information science, statistics and some other fields. Although the total budget of the Defense Advanced Research Projects Agency (DARPA) is due to increase (it supports work on super-computers and other advanced technologies), DARPA's information-processing technology office, which sponsors some fundamental science, will remain at the same level as in 1982, \$75 million.

There are several policy implications of this curious trend. One is that the colleges and universities, if they wish to cash in on the boom in defence funding, will have to seek applied money from the mission agencies for hardware and systems development on things such as the MX missile (see *Nature* 300, 673; 1982). Another consequence is that the administration's defence build-up may be hurt by the lack of corresponding attention to the fundamental work needed to underpin advances in defence technologies.

But the most obvious conclusion is that the science administrators of Secretary Weinberger's defence department have relatively little clout with him, compared with the programme officers who have successfully fought for big spending increases.

Evidence of this came when Weinberger reluctantly agreed to trim \$8,000 million from his fiscal 1984 request of \$280,500 million for defence. The cut took \$17 million away from the original \$867 million request for fundamental defence science. In addition, a modest \$30 million per year, 5-year programme to improve university instrumentation, which was deluged with requests (see *Nature* 27 January, p.277) totalling \$640 million, was continued at the previous level.

However, the relatively flat curves for defence basic research funds may let the new, more independent-minded Congress put its own stamp on defence research.

**Deborah Shapley**

## Australia's new government

# Jones the science

*Canberra*

THE new federal ministry sworn in by the Governor-General on Friday included Mr Barry Jones as the minister for science and technology. The allocation of the portfolio comes as no surprise as he has been the shadow minister since 1980, and has championed the cause of high technology, particularly biotechnology.

Like the Prime Minister, Mr Bob Hawke, Mr Jones has an entry in the *Guinness Book of Records*, but, in the case of Mr Jones, as the world quiz champion outside the United States for 1960–68. (Mr Hawke's achievement involved beer drinking at Oxford in 1955.) Before becoming shadow minister, Mr Jones had been a teacher, lecturer, lawyer and member of the Victorian House of Assembly, before winning the federal seat for Labor in 1977.

Mr Jones has expressed concern for "the winding down of Australia's intellectual capacity", "Australia's extremely adverse position in technological development and transfer", and the diminishing research and development investment by the private sector. His recent book, *Sleepers, Wake!* is an analysis of work in an age of automation.

Although a man of his calibre should improve morale in the Department of Science and Technology, it is a little disappointing that Mr Jones is not a member of the 13-member inner cabinet — a departure from Australian Labor Party tradition — chosen at the caucus meeting last week. Neither has he been invited to participate in next month's national economic summit, to which representatives of government, industry and trade unions have been summoned by Mr Hawke in an urgent attempt to solve the country's economic ills. It seems that Mr Jones has yet to convince his own colleagues of the significant role of science and technology policy in a recipe for economic recovery.

**Vimala Sarma**



## Australian minerals

# Bureau's national involvement signifies policy change

THE Australian Bureau of Mineral Resources, Geology and Geophysics (BMR) is nearing the end of a transformation of its role in Australian geoscience. A sign of the change is the bureau's involvement in a forthcoming national programme of research into the continental crust, for which proposals were discussed at a recent meeting in Canberra (see p.214).

Following a government decision in 1978, BMR has been changed, broadly speaking, from a survey body into a resource-orientated research organization which, with its continental outlook, will act as a coordinating force for basic, resource-related research. Judging by the scope of its research programmes, BMR's nets are to be widely cast over the scientific puzzles posed by the surface and underlying structure of Australasia.

Before its transformation, BMR had been responsible for the geological mapping of Australia at a scale of 1:250,000. In 1978, the Australian Science and Technology Committee (ASTEC) recommended drastic changes and the government agreed. The mapping had been largely completed and, with one of two exceptions (such as the Northern Territory, where support from BMR is still being provided), the maintenance of surveys was transferred to regional bodies. But BMR has been asked that its research, although it might be more fundamental and wider in scope, should have more justification than pure intellectual curiosity. A new director, Dr Roy Rutland, was appointed to carry out the transformation.

The most obvious changes have been in BMR's structure. The bureau now has divisions concerned with geophysics, continental geology, petrology and geochemistry, marine geoscience and petroleum geology, together with the Bass Becking Laboratory and the Special Projects and Geoscience Services (responsible, among other projects, for Antarctic research). Each subdivision has a newly appointed director, and part of Dr Rutland's achievement has been to persuade the Australian Government that a sufficiently high status and salary should be provided for these and other research posts. Most of the scientific staff before the change were at "experimental officer" level; the reorganization of BMR has resulted in a significant

number (70 or so) of posts at the more senior "research scientist" level. Of these, about 45 have been filled by internal promotion.

Although its research must be related to mineral resources, BMR's scientific programme is wide, (as is its primary mandate "to develop an integrated, comprehensive, scientific understanding of the geology of the Australian continent, the Australian offshore area and the Australian Antarctic Territory, as a basis for minerals exploration").

Thus BMR is newly involved, for example, in research into active and passive continental margins (division of marine sciences and petroleum geology) and earthquake seismology and seismic risk (division of geophysics). And a mark of the prominent role to be played by BMR from now

on its the chairmanship by Dr Rutland of the LITSAC project (Lithospheric Transect Studies of the Australian Continent) which was discussed at the Canberra meeting, and the chairmanship of the Australian National Committee for the International Lithosphere Project by Dr Michael McElhinny, director of BMR's division of geophysics.

Inevitably, the transformation of BMR has involved controversy, internal and external. The large amount of "new blood" brought in (particularly at higher levels) created strains within the organization, although these have now been somewhat moderated, partly by early retirements. More visibly, there was disagreement between BMR and the Commonwealth Scientific and Industrial Research Organization (CSIRO) on their future relationship. The situation seems to have been resolved so that CSIRO is to play a tactical role in collaboration with industry on the enhanced exploitation of mineral resources after discovery.

Philip Campbell

## High-energy physics

# Collisions on course to produce shower of charged particles

NOT content with the recent discovery of the intermediate vector boson, physicists at CERN, the European Centre for Nuclear Physics near Geneva, intend to double the energy of the collider that produced the bosons and seek a totally different but equally spectacular phenomenon: Centauro events.

Centauros are collisions involving a high-energy cosmic ray and a nucleus in the atmosphere, in which a vast shower of charged particles (around 100) is produced, but almost no neutral ones. Five Centauros have been detected in cosmic ray experiments, but there is no conventional explanation of these events. One possibility is that they represent a phase transition: that they are the sign of the formation and decay of a quark-gluon "gas ball", a gaseous state of the quarks as opposed to the usual "liquid" state of the photon, neutron and other hadrons.

Noting this, John Rushbrooke of the University of Cambridge a year ago proposed an ingenious way of raising the energy of proton-antiproton collisions in the CERN collider so as to match the probable energy of the Centauro cosmic ray collisions — and so reproduce the events in a controlled way in the laboratory. His proposal, made as part of a Bonn-Brussels-Cambridge-CERN-Stockholm collaboration, has now been accepted by the CERN scientific programming committee.

The problem limiting the energy of the collider is ohmic (electrical) heating in the bending magnets, which steer stored bunches of protons and antiprotons in opposite directions around the super-proton-

synchrotron ring. Fears of overheating have limited proton and antiproton stored beam energy to 270 GeV, and thus collision energies to 540 GeV. But, Rushbrooke reasoned, if the magnet current could be pushed just briefly higher, and for the remaining time held lower than needed to run beams at 270 GeV, it might be possible to accelerate the protons and antiprotons briefly to a higher energy and then drop them down again, keeping the same mean level of heating in the magnets. "It will cost nearly nothing", Rushbrooke says.

This, then, is the accepted proposal: to try out pulsed operation of the collider, with collision energies reaching 800–900 GeV for perhaps 3 seconds in a cycle lasting 14 seconds. The beam would "rest" between peaks at 100 GeV. Experiments should take place in 1984.

The exercise is a gamble, however: a gamble on whether the accelerator can be made to operate in this way without losing too much beam (a loss of 1 per cent per cycle would be just tolerable) and a gamble on whether the energy is right. Rushbrooke estimates that 5–10 per cent of some 30,000 events collected in a few days' operation should be Centauros; but the measurement of cosmic ray event energies is so inaccurate that it is possible that the threshold (minimum energy required) for the Centauros is even higher than Rushbrooke can reach. If so, it will be bad luck; if not, CERN will have beaten the United States to physics which will become available at the 1,800 GeV (centre of mass) collider at Fermilab, near Chicago, only by late 1986.

Robert Walgate

## British Technology Group

CONTRARY to the suggestion made in *Nature* last week (10 March, p.100), Dr Richard Flavell has not in fact been offered an appointment with the new agricultural genetics company proposed by the British Technology Group. We apologize for any embarrassment caused.



## Drug safety

# No cure for headaches in the pharmaceutical industry

PUBLIC disquiet about drug safety is unlikely to be lessened by last week's developments in Britain. First Zomax, a widely prescribed painkiller, was withdrawn from sale following five deaths in the United States which appear to be linked with the drug. Later, a report published by the Consumers' Association criticized the safety record of Distalgesic, by far the most commonly prescribed analgesic of its type in Britain. So soon after the publication of the Greenfield Report, which encourages more generic prescribing, these events could hardly have come at a worse time for the pharmaceutical industry.

Zomax (zomepirac sodium), manufactured by a subsidiary of Johnson and Johnson, is a non-steroidal anti-inflammatory drug introduced in Britain in 1980. Since then, an estimated 20 million people worldwide have taken the drug. Its distributors in the United States, McNeil Pharmaceuticals, first became aware of anaphylactoid shock reactions in some patients last year. Two patients in the United States known to be hypersensitive to

aspirin died, as a result of which prescribing advice was altered to emphasize the unsuitability of the drug for patients with aspirin or other anti-inflammatory induced allergy. Another three deaths this year, still under investigation, prompted McNeil voluntarily to withdraw Zomax from the US market while further labelling changes are considered.

The distributors of Zomax in Britain, Ortho-Cilag Pharmaceuticals Ltd, followed suit after consultations with the Committee on Safety of Medicines. Dr Wendy Jefferson, medical director of Ortho-Cilag, points out that at least until last month, no deaths associated with Zomax are known to have occurred in Europe.

Distalgesic is another matter. The Consumers' Association report, published in its *Drugs and Therapeutics Bulletin*, asserts that Distalgesic is the drug most commonly associated with suicidal and accidental drug death in Britain even though it is a prescription-only medicine and so less widely available than other potentially fatal drugs. The report pins the blame on a component of Distalgesic called dextropropoxyphene (DP), an opioid analgesic. Some doctors feel that the efficacy of Distalgesic owes something to a psychotropic effect of DP, and its use is banned in some hospitals. The Consumers' Association report concludes that there is insufficient evidence that the combination of DP and paracetamol contained in Distalgesic is more effective than paracetamol alone, and that "uncritical" use of the drug seems "pointless and hazardous". DP overdose causes rapid death through respiratory failure, and the suggestion appears to be that the safety margin between the recommended therapeutic dose and a lethal dose is too narrow.

The coroner of the city of Birmingham, Dr Richard Whittington, estimates that up to 500 deaths a year may be caused in Britain by Distalgesic, especially when it is taken with alcohol.

Distalgesic is manufactured by Dista Products Ltd. Dr Wilson Totten, medical adviser for the company, denies that the safety margin of Distalgesic is lower than that of other centrally-acting analgesics, and refutes suggestions that mild dependence on Distalgesic may be widespread.

Dr Totten claims the evidence presented in the Consumers' Association report is incomplete, and criticizes its authors for distributing copies to the lay press at the same time as submitting it to the regulatory authorities. The Committee on Safety of Medicines has now decided to "consider whether there is any new evidence" bearing on the safety of Distalgesic.

Tim Beardsley

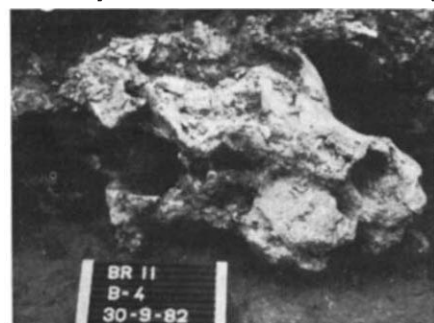
## Belgian archaeology

# Neanderthals hit

Brussels

THE grave financial problems of the University of Liège are threatening excavations at a site which promises to reveal new evidence about the evolution of man in Europe. The site, at Belle Roche some 30 km south of Liège, was discovered three years ago by a quarryman who noticed some fossil fragments in a band of lime deposits. Intense excitement was aroused last year, however, when a team from the University of Liège discovered stone tools which may have been made around 500,000 years ago.

Dr Jean-Marie Cordy, a lecturer in palaeontology at the university who has taken charge of the project, thinks that the discovery of the tools indicates a strong



The well preserved cranium of a deninger bear from Belle Roche

possibility that skeletons of pre-Neanderthal man will be discovered in the next few years. Many plant and animal remains from the middle Pleistocene period have already been identified, including the well-preserved craniums of a deninger bear and a European jaguar. Belle Roche is expected to become one of the key sites for tracing the origins of European man in the period stretching from the appearance of *Homo erectus* 1.5 million years ago to the arrival of modern man (*Homo sapiens sapiens*) less than 50,000 years ago.

Unfortunately, the financing of such research is poorly organized in Belgium, and the company that owns the Belle Roche quarry cannot much longer postpone mining the face where the relevant limestone is being excavated.

The university has been able to find the BF400,000 (£5,200) that the excavation has so far cost, but is now in such dire financial straits that no more money will be forthcoming. Future excavation would require BF2 million a year. The most pressing need is for a permanent staff of researchers. The dozen or so workers engaged on the actual digging are mostly redundant steelworkers whom the state partly pays to undertake socially desirable work.

For 1983, the financial problems, apart from paying staff, have now been solved thanks to the timely intervention of the Fonds National de la Recherche Scientifique (National Science Research Fund), but the research cannot be managed on a hand to mouth basis.

Jasper Becker

# Super-Sara obituary

Brussels

THE Super-Sara reactor safety project at the European Community's joint research centre at Ispra in Italy was quietly abandoned last Thursday at the meeting of the Ten's research ministers. Italian objections were overcome by assurances that spending levels would be maintained and that every effort would be made to site the next big fusion machine at Ispra.

The debate on the European Commission's proposals for research projects to take the place of Super-Sara revolved around a tritium-handling laboratory, which in the non-military field is lacking in the European fusion effort; an earthquake simulation machine intended to find out how well large nuclear and non-nuclear installations would stand up to catastrophic impacts, and feasibility studies on either Ignitor, one of the alternative fusion machines to the tokamak, or NET (the Next European Torus).

More discussions will take place throughout the year, but one thing is certain: the Commission has a budget of 700 million European Currency Units (ECU) (£1,500 million) to spend on research between 1984 and 1987 and 2,260 people to occupy. The council decided to allocate 20 million ECU partially to decommission the Essor experimental reactor, half of what the Commission had originally estimated was needed, which suggests that further uses will be found for it.

Jasper Becker

## Nuclear power technology

# Liberal policy plea for free trade

INTERNATIONAL trade in nuclear power technology could make a major contribution to international security, Mr Ian Smart, an international energy consultant, told the Royal Institute of International Affairs in London recently. Since the detonation by India in 1974 of a nuclear explosion using plutonium from a heavy water reactor supplied by Canada, international trade in nuclear power technology and materials has become increasingly a matter of unilateral and sectional regulations by supplier countries and at the same time, said Mr Smart, a cause of "general alarm and growing despondency". Had a liberal policy of nuclear trade continued, he argued, the situation now would be less gloomy.

Mr Smart argued that nuclear power is dangerous "only insofar as all human activity is dangerous". It cannot be ignored, at least for the next 20 years (by which time, at the "most pessimistic" estimate, nuclear plant will account for at least 20 per cent of the world's total generation capacity). Although it is "ludicrous" he said, to "equate nuclear power with nuclear weapons", the Indian explosion showed that nuclear power inevitably spreads nuclear skills and materials, including such sensitive technologies as enrichment. By the year 2000, it is estimated, some 38 countries will possess reactors, and enrichment technology will inevitably have proliferated.

Since 1974, the supplier governments have imposed their own unilateral constraints on the sale of nuclear technology, producing a situation in which a would-be purchaser can shop around to find the least

demanding supplier, and, failing that, will try to go it alone, producing reactors which are at once less efficient and less safe.

Smart argued that for economic reasons as well as safety, it would be more satisfactory to have relatively few fuel-cycle plants (which would therefore be under international control). The post-1974 atmosphere of distrust, Mr Smart argued, is a result of the lack of a liberal policy of trade plus safeguards and the failure to develop a sound basis for non-proliferation of weapons before 1974, for which the "nuclear weapons states are primarily responsible". For the original concept of the Non-Proliferation Treaty of 1968 was that preferential trading terms should be given to customer states that acceded to the treaty, accepting agreed "full-scale" safeguards against the diversion of the transferred technology to weapons. Such a system would make the treaty attractive to all states except those which deliberately wanted a nuclear weapons programme, making it possible, at the very least, to pinpoint potential offenders. At present, however, says Mr Smart, there is a good argument for non-nuclear-weapons states to stay outside the Non-Proliferation Treaty, which entails international control with full-scale safeguards and no great incentives. But countries outside the treaty can still purchase nuclear technology without the fuss of full-scale safeguards, and such bilateral agreements are not conducive to safety. The Indian explosion of 1974 contravened neither safeguards arrangements nor treaty obligations but exploited a loophole in the purchase agreement of 1955.

Vera Rich

## Chelsea physic garden blooms

ONE of Britain's oldest botanic gardens, the Chelsea Physic Garden, which was founded in 1673 by the Society of Apothecaries for the study of plants for "medicinal and general scientific use", is to be opened to the public this year for the first time in over 300 years. It was this four-acre garden in London that supplied the cotton growers of Georgia in the American South with their original seed stocks. Other exports include Chinese bananas to Fiji and rubber to Malaysia.



## US national laboratories

# Leander Argonne looks ahead

Argonne, Illinois

NOT very long ago, things did not look good for Argonne National Laboratory. The boom of Carter-era solar and conservation research had collapsed with the coming of the Reagan Administration; 800 jobs were being eliminated; grumbling was heard in high places that Argonne had strayed from its original mission into a maze of unrelated and undirected projects. And with the more glamorous high-energy physics research and new accelerator projects passing Argonne by, a hint of woebegoneness was beginning to settle over this laboratory founded in the glory days of nuclear physics that followed the Manhattan Project.

Now, after two years of cutbacks and no small amount of rethinking by its director, Dr Walter Massey, Argonne seems poised for a comeback. "There were things that, when I got here" — in 1979 — "I wondered why they were being done", Massey says. He stopped some of them, reemphasized Argonne's traditional strengths, and did not shy away from initiatives that fit nicely with the administration's philosophy on government cooperation with industry.

The most obvious of these efforts came to fruition last October, with the repackaging of Argonne's historically strong materials science expertise into a more coherent and more visible programme. Work that had been scattered throughout the laboratory was brought together into a new Materials Science and Technology division that will concentrate on defects studies, surface science and corrosion, and advanced materials research.

Dr Brian Frost, director of the division, says the reorganization has already reaped scientific benefits by breaking down disciplinary barriers. A research project on organic conductors, for example, has gained new life, he says, now that physicists as well as chemists are involved.

The political benefits are sure to be great, too. Materials science has become the prototype of research that this administration believes government belongs in: too applied for universities, too risky for industry. Worries about Japan, where industry and government are investing heavily in ceramics research and worries about supplies of strategic materials have added to the appeal of materials science in the American political realm.

Lawrence Berkeley Laboratory (LBL) recently unveiled plan to establish a National Center for Advanced Materials received the enthusiastic backing of the President's science adviser, Dr George Keyworth; Argonne can no doubt count on similar support.

While LBL's centre will be built around a new synchrotron light source, Argonne



will have as its cornerstones its new Intense Pulsed Neutron Source (IPNS) and high-voltage electron microscope. Argonne will have access to synchrotron radiation with its own beam-lines at Brookhaven and the University of Wisconsin.

While Massey says there is no competition between Argonne and LBL over materials research, LBL's initiative and the fanfare surrounding it has clearly rankled. Dr Kenneth Klierer, director of physical research, jokes that he may have to come up with an impressive name for Argonne's materials division. And Frost says that the research community is "a little uneasy" that it was not consulted on LBL's plan.

But materials research at Argonne cannot in any case play the starring role that it will at LBL. LBL's materials centre will eventually involve one-third of the laboratory's staff and budget. At Argonne, which is two-and-a-half times the size of LBL, "it's more difficult to have a single effort on which to base our future", Massey says. "We're trying to do good research — that's the best assurance of our future."

One area in which Argonne has traditionally done good research is nuclear physics. Argonne's programme suffered a blow several years ago with the shutdown of the Zero Gradient Synchrotron. The laboratory is only now regaining its footing, most notably thanks to the success of IPNS, a project that was almost killed not long before it was due to be completed. "Two years ago there was a large number of people saying there never would be one neutron coming out of IPNS", Klierer says. Since it went into operation, 120 experiments have been run by groups from 50 different institutions. "This was a situation where the external perception was so negative, and the reality was exemplary".

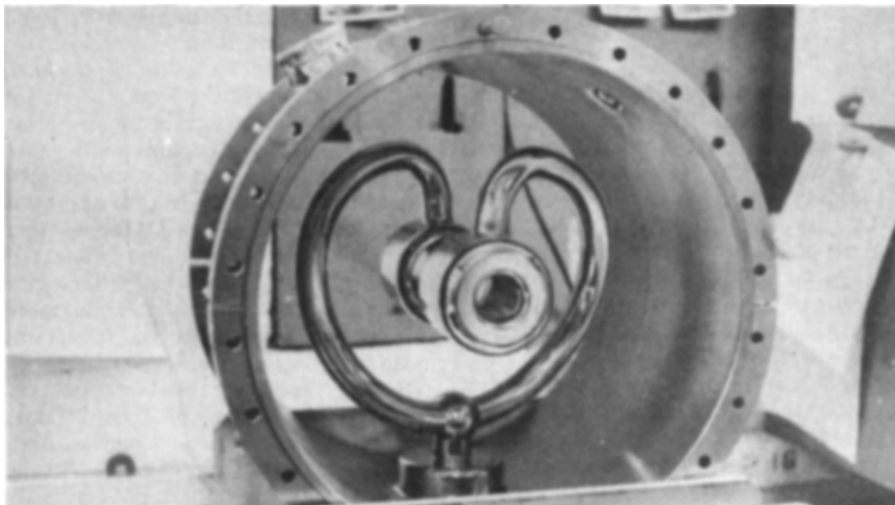
Argonne's comeback is also evidenced



Argonne director, Walter Massey.

by its being the site of one of the very few nuclear physics construction projects now under way. ATLAS (Argonne Tandem Linear Accelerator System), an expansion of the existing tandem ion accelerator, is due to be completed in 1985. By adding superconducting of resonators, its peak energy will be boosted from 10 MeV to 25 MeV, allowing ions as heavy as the tin isotopes to be accelerated.

Argonne also appears to be leading the competition for a new electron accelerator. Argonne's proposal, GEM (GeV Electron



One of the superconducting R.F. resonators for ATLAS

Microtron), is a 4 GeV accelerator to be built on the site of the abandoned Zero Gradient Synchrotron. Argonne is vying with a consortium of south-eastern United States universities for the construction contract, and appears to have the lower bid. A subcommittee of the Energy Department's Nuclear Science Advisory Committee is to make a recommendation on 20 April on the choice of proposals.

The electron accelerator is likely to be the centre of a minor revitalization of nuclear physics, which in recent years has seen more and more of the "glamour" move to high-energy physics by probing nuclear phenomena at short distance, where quark-gluon dynamics are expected to show up. "Nuclear physics is really going to get a lot more attention and visibility if they fund this machine", Massey says. And Argonne, with its traditional strength in nuclear physics, would like to be at the centre of this revitalization.

Argonne's concentration on a third traditional strength, nuclear engineering, is more of a gamble than materials or nuclear physics as a basis for the laboratory's future. Although nuclear reactor development, particularly of the breeder reactor, is enjoying the strong support of the administration, Congress has repeatedly come within a hair's breadth of axing the most prominent part of the programme, the Clinch River Breeder Reactor demonstration project. "A large part of the research here is in an area that is politically sensitive", Massey concedes, although he believes that even if Congress decides that now is not the time to build Clinch River there will be support for a base programme". The issue is likely to be the size of that base research programme rather than its existence.

Argonne has had hard times in this area before. At its founding in 1946, the laboratory took the lead in the Atomic Energy Commission's effort to develop peaceful uses of atomic energy. Virtually all fission power systems now in use or planned were developed at Argonne. But in the mid-1960s, the government decided that with nuclear power well on the way to

commercialization, it should no longer be involved in developing light-water reactor technology.

The laboratory was able to cover itself then by shifting its activity over to the breeder; it may be able to cover itself again, if the breeder programme is cut, by shifting over to fusion reactor engineering. The laboratory is already at work on preliminary studies of nuclear systems for a fusion reactor, such as the reactor blanket and magnets; and, as Eric Beckjord, Argonne's deputy director, notes, "Fusion reactors are going to take a lot of nuclear engineering and materials R&D to come out with something that's practical".

A smaller although probably more stable source of funding for the reactor division is the Nuclear Regulatory Commission, which contracts for studies relating to reactor safety and regulatory issues. At Argonne's facility in Idaho, the laboratory maintains several research reactors designed to answer safety questions relating to breeder reactors and applicable to light-water reactors as well.

Argonne, perhaps more than the other national laboratories, has suffered in recent years from "roller-coaster" funding, most notably in the boom and bust of solar energy research. Massey, commenting on Congress's investigations of waste at the laboratories, says "when they come to look at waste, fraud and abuse, the biggest waste is those big fluctuations in funding". The recent assessment of the national laboratories by the Energy Research Advisory Board recommended multi-year funding authorization for the laboratories to help smooth out such sudden changes.

But the laboratories' diversity has clearly been at least partly responsible for this problem, a point that will reportedly be underscored in an assessment by the Office of Science and Technology Policy due shortly. Argonne hopes that its decision to concentrate on a smaller number of projects — and on projects perhaps less susceptible to the changing mood of Congress — will anticipate this criticism, and assure itself of a more stable future.

Stephen Budiansky

## No PWR, thanks

SIR — The leading article "Inventing the wheel" (*Nature* 300, 672; 1982) about the rights and wrongs of holding a public inquiry into the possibility of a Sizewell-based pressurized water reactor (PWR) seemed to be derived from information received from the top of Mt Sinai to which the rest of us are not privy. In singling out the Friends of the Earth, or any other member of the opposition to the Central Electricity Generating Board's (CEGB's) proposals, for a rather sneering attack, the writer of the article demonstrated all the sporting sensibilities of a piranha. The opposition to PWRs is widespread and finds its voice through a number of mainly poor subscribed to organizations like the Friends of the Earth. The fact the opposition collectively and individually is fiscally impoverished, however, should not be confused with the intellectual merits of the opposition's many and frequently very disturbing arguments. These arguments relate not only to safety but to economics and conservation issues also. The inquiry is interested in Sizewell not on its own but in the context of a very expensive commitment to a national nuclear power programme.

With reference to the economics of the case alone, three independent bodies have been severely critical of the CEGB's projections. These are the House of Commons Select Committee on Energy, the Monopolies and Mergers Commission and the Electricity Consumers' Council. In addition, there has also been criticism of safety aspects of the proposed Sizewell PWR by the Nuclear Installations Inspectorate. Surely there is a benefit to be accrued from a public forum where the CEGB may be seen to answer such powerful critics.

I must confess I prefer this approach, for example, to the empirical one on the safety aspect advocated in your article where it is recommended that we build a PWR to see what, if any, the dangers are. I have a particular reason to reject this argument as I live in Winfrith, Dorset, in the vicinity of a site under consideration by the CEGB for a future PWR. I wonder if the writer of the editorial would be such a powerful advocate of the empirical method if he lived in my neighbourhood. BARRIE PEARSON  
*Winfrith, Dorset, UK*

## Generic prescribing

SIR — Your report on UK drug prescriptions (*Nature* 17 February, p.557) seems to overlook the fact that generic prescribing has been taught to medical students by university departments of pharmacology and therapeutics for at least 30 years.

Moreover, the Department of Health and Social Security regularly distributes free of charge comparative bar-charts showing very clearly the relative costs per unit of active principle of the different drug preparations with the same therapeutic action which are available, so there is really

no excuse for doctors being unaware of the economics of prescribing.

Why, then, do they not choose the most economic alternative? Certainly relative assonance or dissonance of the name has some influence. The commercial designer of a drug always gets in first with his proprietary name, long before the approved name is devised, and here it is the case that the devil gets all the best tunes. I must confess to finding that the proprietary "Chloromycetin" comes to mind more readily than the approved "chloramphenicol", for instance.

Perhaps the effect of real differences in efficacy and efficiency between different preparations of the same drug has been under-estimated. It is well known that the efficiency and efficacy of drugs is critically dependent on pharmaceutical formulation and process technology. It is in this sphere that most scope exists for differences between the products of different drug firms, and prescribing doctors, who have to live with their therapeutic failures as well as their successes, are not as susceptible to commercial blandishments as BBC television's "Panorama" team would have us believe. They may be expected to choose the preparation which they think will most benefit their patients — perhaps on very limited evidence.

What is needed is very much more attention, at ministry level, to comparative studies of efficacy, rather than active principle content, in relation to cost. If such information were more readily available, it might be expected not only to stimulate rational and cost-conscious prescribing, but also to stimulate the drug firms to produce products which represent real advances on those of their competitors. R.J. ANCILL  
*Pharmacology Department,  
University of Bath, UK*

## Tank makers note:

SIR — As an opponent of the neutron bomb, I welcome the calculations of Seifritz *et al.* (*Nature* 299, 390; 1982), from which a specification for a practical radiation shield for tanks can be derived.

In the following calculations, the shield is described as  $\text{g cm}^{-2}$  (taking weight as the limiting factor) and the weight is kept within the limits adopted by Seifritz *et al.* (around  $60 \text{ g cm}^{-2}$ , the weight of 5 cm steel plus 10 cm water plus 1 cm lead plus the layer of boron or  $\text{Li}_2\text{O}$ ).

Assuming that the effect of a moderator material on neutrons depends only on its hydrogen content, I propose as moderator material an aqueous solution of ammonia saturated at  $0^\circ\text{C}$  and atmospheric pressure, enclosed in a pressure-tight vessel within this shield. A  $7.83 \text{ g cm}^{-2}$  layer of this solution should have the same effect on neutrons as a 10 cm layer of water ( $C_n = 3.2$ ); the effect on  $\gamma$  radiation is assumed to be the same as that of water ( $C_\gamma = \sim 1.5$  per  $10 \text{ g cm}^{-2}$ ). Further, I propose to replace the 2 cm layer of lead as well as the boron or  $\text{Li}_2\text{O}$  by

moderator liquid. It then becomes possible to incorporate into the shield three layers of moderator liquid of  $7.93 \text{ g cm}^{-2}$  each ( $= 23.5 \text{ g cm}^{-2}$ ) with a total shielding factor of  $C_n = 32.7$  or  $C_{\text{tot}} = 13.7$ . Together with the effect of 5-cm steel, protection factors of  $C_n = 63.5$  and  $C_{\text{tot}} = 20.9$  result. The addition of  $7.83 \text{ g cm}^{-2}$  of moderator liquid would make the overall shielding factors  $C_n = 203$  and  $C_{\text{tot}} = 34.4$ , while the weight would still be well below that of 10-cm steel, accepted by Seifritz *et al.* as "the classical armour of tanks".

Considering that the intensity of radiation decreases with the square of the distance from the explosion point of the bomb (a factor ignored by Seifritz *et al.*), the number of tanks ( $x$ ) which can be stopped by a given bomb will be reduced to  $x/C$  by equipping the tanks with a radiation shield of shielding factor  $C$  (equal distribution of the tanks over the area presupposed). Thus where one formerly could hope to stop 100 unprotected tanks, a shield of shielding factor  $C_{\text{tot}} = 20.9$  would reduce the loss of tanks to only 5, making the neutron bomb unfit as an antitank weapon.

Builders of tanks, keen to keep the weight of their vehicles low, might make it possible to jettison the heavy moderator liquid as soon as contact with the enemy became so close that the use of neutron bombs was unlikely. Diesel fuel might serve as a moderator and hence fuel reserve, making the main fuel tank lighter.

ERNST MECKE

*Helsinki, Finland*

## Him or her

SIR — The correspondence on the position of Chinese surnames and the modern practice of hyphenating the following given names which J.A. Irving (*Nature* 299, 200; 1982) and M.C. McKenna (*Nature* 300, 212; 1982) discuss has raised problems of unusual nomenclatural practice. Dr McKenna states that there "is no longer a problem in English translation because given names are now hyphenated and the second one is not capitalized". P.J. Steward (*Nature* 300, 680; 1982) illustrates that the problem exists, at least in sinicized European names. For foreigners, however, the gender of Chinese names and forenames remains confusing. There is a Dr Joan Lai-Fook on the staff of my department; she is Chinese. Obviously her gender would be no problem to Dr McKenna as long as he assumed her surname to be "Lai-Fook". If he assumed that "Joan" was her surname, then it would be logical to assume that she was married to a Dr Joan as Lai-Fook appears to be a given name. In fact, she is Dr Lai-Fook, and her family surname was Lai, to which the given name Fook was added, and Joan is her given name. There will always be exceptions to every rule.

C.S. CHURCHER

*University of Toronto,  
Ontario, Canada*



# Resurrecting a nuclear accident

*A British report has given a fascinating account of a nuclear accident a quarter of a century ago — and in the process pointed to some of the steps that should be taken when and if such events recur.*

THE most spectacular nuclear reactor accident described in any detail so far is not, as may be thought, that at Three Mile Island in the United States but, rather, that at Windscale (now called Sellafield) in the north of England in October 1957. On that occasion, a fire broke out within an air-cooled graphite moderated reactor (of a type designed solely for the production of military plutonium). Over the course of two days or so, a very large amount of radioactivity in the form of volatile fission products was released to the atmosphere and deposited over much of England and part of mainland Europe. The fire was eventually put out by flooding the reactor with water, and the whole antiquated system has now been sealed off. Now, more than a quarter of a century after the event, the National Radiological Protection Board (a formally independent public body concerned with radiological safety in the United Kingdom) has produced a retrospective assessment of the damage done.

The document is valuable principally as a model of how such investigations may be carried out. It has, however, caught the public interest in Britain because of its explicit estimate of the numbers of people who would be expected to have contracted thyroid cancer as a result of the accumulated dose of radiation from iodine-131. Briefly, the authors of the report have estimated, iodine-131 from Windscale should eventually be responsible for 260 cases of thyroid cancer, of which (given standard treatment) about 13 should be fatal. The incidence of thyroid cancer works out at about 1 per cent of that from natural causes, and so is statistically undetectable.

It is, however, honest, even courageous, for a public establishment to put out a document acknowledging not merely that radioactivity from a nuclear reactor can kill but that it has killed people. This confession, long overdue, will no doubt feature in one form or another at the public inquiry into the proposal to build a pressurized water reactor in East Anglia, now under way. In the long run, however, it should contribute to a sense of realism about the hazards of reactor accidents.

As a model calculation of the consequences of reactor accidents, the retrospective analysis of the Windscale accident is chiefly of interest in directing attention to the gaps in the data collected in the days and months after the accident. The pro-

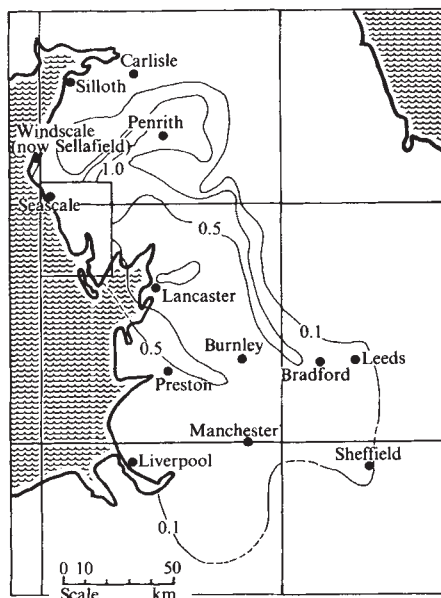
cedure, while tediously complicated, is in principle straightforward enough. The objective is to infer from the data that exist about the distribution of radioactivity the collective dose commitment to the population at risk from the radioactivity released (still chauvinistically measured in units of man-Siverts, with 1 Sievert in the SI system equivalent to 100 rem in the old system). There are, of course, some immediate complications: the collective dose commitment (in the sense of the total exposure of the whole population at risk, past and future) must be calculated separately for the particular exposure of the thyroid as a consequence of ingested iodine-131 and for the general exposure of the body as a whole. Then it is simple to look up the dose-response relationships so as to calculate the likely damage.

The more serious errors in the analysis are not however those implied in the dose-response relationship, not even in the correction of the latter to allow for the age

of iodine-131 accumulating in a person's thyroid would have been determined not only by his or her milk-drinking habits but also influenced importantly by the decision of the authorities not to allow the distribution of milk from the neighbourhood of Windscale for days or even weeks (according to distance) after the accident. Fortunately, it has been possible to compare the estimates with a few experimental measurements of iodine-131 concentrations in the thyroid glands of actual people both near Windscale and in the more distant cities of Leeds and London. The numbers (a dozen or so) are too small for the measurements to be significant, but at least they are in the same ball-parks as the estimates, which is in the circumstances remarkable.

Even so, the remaining errors are large and their pattern is curiously skewed. The authors of the report estimate that three-quarters of the collective thyroid dose commitment from iodine-131 released in 1957 arose in the population then within 200 km of the damaged reactor, a region in which direct measurements of iodine-131 in milk were, at the time, systematic and comprehensive. The dose commitments arising from other fission products, two strontiums and two rutheniums for example, are lesser health hazards (perhaps a quarter of the total) but also much less easily calculable because of the need to estimate the rate of transfer into people's bodies on the basis of estimates of deposition velocities and the like. Overall, the authors estimate that their result is probably correct within a factor of two, but that may be optimistic.

Nevertheless, the result has important implications for those whose responsibility it may be to superintend other nuclear accidents. The simple moral seems to be to measure everything in sight. The problem is that of mounting in a few hours an investigation of the kind normally extending over several years required to fix standards for the release of radioactivity in the routine operations of nuclear plants. A quarter of a century ago, the people at Windscale seem to have performed creditably, especially in guessing reasonably well the distance at which to make measurements. □



Contours of  $^{131}\text{I}$  deposition in Northern England in 1957 in units of microcuries  $\text{m}^{-2}$  (1 microcurie is roughly 37,000 Becquerel)

distribution of the exposed population but in the estimate, necessarily only approximate, of how many people were exposed to how much radiation from which different sources.

At Windscale in 1957, the distribution of radioactivity in the neighbourhood was complicated by the shifting wind over a period of two days or so, while the quantity

M.J. Crick & G.S. Linsley. An assessment of the Radiological Impact of the Windscale Reactor Fire, 1957. (National Radiological Protection Board Report 135, HMSO, 1983; £3.00).

## Ho Chi Minh City conference

# Defoliants in Vietnam: the long-term effects

from Alastair Hay

VIETNAM'S inland forests still bear the scars of a 10-year herbicide spraying programme by the US Air Force during the Vietnam war. Twelve years after the spraying ended in 1971 there has been little regrowth of secondary forests in areas sprayed three or four times. Some of the mangrove forests, of which about 36 per cent of the country's total acreage was destroyed by herbicides, are being restored, however, in a massive replanting programme begun in 1977. Of the 8,000 hectares reseeded in one area close to Ho Chi Minh City (formerly Saigon), over 60 per cent is now firmly restocked with young mangrove trees.

Successful though this one replanting programme appears to be, the area restocked only represents a small fraction of the total devastated by the spraying programme. The difficulties encountered in the replanting of the mangroves and the failure of the tropical inland forests to regenerate were two of the topics discussed at a recent symposium\* in Vietnam.

The symposium was organized with the purpose of evaluating the long-term effects, on man and his environment, of the military spraying programme during which, according to official US figures<sup>1</sup>, some 10.3 per cent of Vietnam's inland forests, 36.1 per cent of mangrove forests, 3 per cent of cultivated land and 5 per cent of other land was sprayed. Some 90,000 tons of herbicides and anti-plant chemicals were used between 1962 and 1971. Most of the spraying was with Agent Orange (a 1:1 mixture of 2,4-D and 2,4,5-trichlorophenoxyacetic acid), Agent White (2,4-D and pictoram) and Agent Blue (cacodylic acid).

Delegates to the conference were taken on one, or several, trips into the field to assess the forests at first hand. From this visual evidence and by matching aerial photographs with military spray records, it seems clear that the extent of permanent damage to the secondary inland forests is well correlated with the concentration of herbicide used. The degree of initial damage and subsequent rate of recovery, however, depend on many factors, including the species of vegetation, herbicide concentration, total area affected (smaller areas will usually recolonize much faster), the terrain, soil fertility, soil micro-organisms and weather.

The existence of a prolonged dry period in Vietnam has impeded regeneration of the inland forests, and replanting will be essential. Human intervention in some of the defoliated areas has meant that some of

the forest areas are now farmed. This will be a difficult policy to reverse and it was the view of one of the symposium working groups that this land is probably best left to agriculture.

Fires are another major problem. The burning of grass in the defoliated areas also has had its effect on the recolonizing of the area and would have killed young trees. Fires are a recurrent threat for foresters, but more fire breaks and closer management will help to control the situation.

Repeated application, as opposed to a single spraying, of herbicides has clearly resulted in more destruction of the forest and conversion of the land to other use. The fertility of the soil falls if forests are replaced with grassland or bamboo, as has happened in much of the sprayed region. In these regions there has been a loss of minerals and nitrogen and in some cases a fall in the pH of the soil. All these changes will inhibit recolonization. What is urgently required is the development of techniques to restore fertility. Help is being solicited for a programme in this area.

An accurate inventory of the extent of the defoliated forests, inland and mangrove, is a further urgent priority to assess the factors which have allowed, or prevented, recolonization. In some of the mangrove forests, regrowth has not occurred because of the total destruction of the seed source. The Rung Sat area near Ho Chi Minh City had been reduced to mud flats by 1972, but a replanting programme begun in 1977 using seedlings imported from the Cau Mau forests 300 miles to the south has restored some of the area. Young trees are growing on soil previously defoliated, and the local fishing industry, devastated by the destruction of the forests and consequent silting of the tidal water, is beginning to recover.

Vietnamese scientists remain convinced, and concerned, that defoliation has not only led to changes in local rainfall and excessive erosion, but may have caused irreparable damage to the local fauna and flora. As far as inland water ecosystems are concerned, Vietnamese scientists report that defoliation has led to a loss of freshwater vertebrates and invertebrates and changes in the composition of the local algae.

Defoliation has clearly affected the wildlife, both in the areas which were totally defoliated and subsequently converted to grassland, and in those regions less frequently sprayed where the plants in the upper reaches of the forest were destroyed. In both areas, the number of animals and birds has dropped dramatically. Figures were given for one census in the A Luoi valley in Binh Tri Thien province (formerly

Quang Tri). The valley was heavily sprayed and only 24 species of birds and 5 species of mammals were observed, whereas in two control forests there were respectively 145 and 170 bird species, and 30 and 55 mammalian species. Animals are also at risk in some of the forest areas which have been isolated from the main body of the forest by the spraying. It will be essential at least to grow corridors of trees to try and re-establish the connection if the animals are to be saved.

Much of the controversy about the spraying programme is concerned with the health of the people exposed. In particular, there is concern that one of the herbicides — Agent Orange — had a high concentration of a contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin). Dioxin is teratogenic<sup>2,3</sup> and carcinogenic<sup>4,5</sup> in animals, and there is understandable concern that it may show these effects in humans.

Using US official figures, it is possible to calculate that some 170 kg of dioxin were deposited on Vietnam<sup>1</sup>. How much remains is a moot point. Measurements of dioxin in fish collected in 1970 registered levels of 800 parts per 10<sup>12</sup>. Some recent soil samples, analysed in Holland, had levels up to 30 parts per 10<sup>12</sup>. But it is not clear how representative of the sprayed region these soil measurements are as too few have been made and there is no information on the movement of dioxin through the ecosystem.

As for specific health effects in people exposed to herbicides, Vietnamese scientists presented evidence for an increase in primary liver cancer, spontaneous abortion, birth defects and chromosome aberrations. Dioxin exposure is believed to be a factor leading to these increases.

It was the opinion of the conference that there was some suggestion of a link between herbicide exposure and primary liver cancer. Before anything definite can be said on this question, however, further studies are needed on a large population. In subsequent investigations more details will be required on the extent of individual exposure to herbicides, the body burden of dioxin, the role of hepatitis B in events, and results of objective laboratory tests. Larger control populations are also needed and reporting bias must be avoided.

Some of these requirements also apply to future investigation on the outcome of pregnancies in which one or other spouse was exposed to herbicides. One investigation that appears to have been well conducted suggests that where fathers have

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\*The international symposium on herbicides and defoliants in war: the long-term effects on man and nature\* was held in Ho Chi Minh City on 13–20 January 1983. Conference proceedings are to be published by the Stockholm International Peace Research Institute.



been exposed to herbicides their wives have had more pregnancies where the outcome was unfavourable, that is spontaneous abortions and child born deformed, than was observed in a control population.

The increase in chromosome aberrations and sister chromatid exchange observed in adults exposed to herbicides and in their children was assessed using standard non-banding cytogenetic techniques. As the published literature on this subject is extremely controversial<sup>6</sup>, it was felt that these findings required urgent confirmation in other laboratories.

Much of the work presented by Vietnamese scientists at the conference was in many ways only a first look at the problem.

## Supernova

# Carbon detonation redux

from J. Craig Wheeler

A DECADE ago carbon detonation was all the rage among supernova theorists. The idea was that the characteristic burst of light and other radiation that astronomers label a type I supernova came from a star whose core of degenerate carbon suddenly underwent explosive nuclear burning. The situation was thought to arise in highly evolved stars of mass 4–8  $M_{\odot}$  after they had lost their hydrogen envelope in the stellar wind.

The notion that 4–8  $M_{\odot}$  stars were the site of the 'carbon detonation supernova' permeated the general astronomical community at about the time the experts were deciding that carbon probably does not detonate and that 4–8  $M_{\odot}$  stars may not ignite carbon anyway. However, new concepts of the nature of type I supernovae and novel numerical procedures have brought the question of the nature of degenerate carbon ignition to the forefront of supernova research again. In a recent paper, the first hydrodynamical calculations of carbon models are carried out by W. David Arnett, and Ewald Muller makes an important new contribution towards understanding the complex problem of degenerate carbon ignition (Muller & Arnett *Astrophys. J. Lett.* **261**, L109; 1982). This new work reinforces the idea that degenerate carbon ignition will lead to the subsonic combustion of a major fraction, but not all, of the star and to complete disruption with no neutron star remnant.

In the mid 1970s the realization slowly came that highly degenerate carbon (that is, a highly compressed form of carbon that departs from the classical equation of state in its behaviour) would not burn by means of a self-propagating supersonic detonation wave. This conclusion did not come easily because a detonation will propagate in appropriate stellar conditions once it is initiated. The problem is that the Fermi energy in carbon at densities in excess of  $10^9 \text{ g cm}^{-3}$  is so high that the ignition and in-

More work is required to validate their findings and cooperation on an international basis to facilitate the programme is being sought. International aid agencies, such as WHO and those in the UN concerned with the environment, have an important part to play and will be called upon to help. □

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cineration of a small portion of the centre of the star does not provide enough overpressure to drive a sufficiently strong shock. The conclusion is strengthened by consideration of geometrical factors in the spherical expansion of the small central portion where ignition begins. It appears, rather, that degenerate carbon, though extremely volatile, burns by means of a subsonic front which propagates by turbulent exchange of heat between incinerated and unburned matter.

Few modern problems in stellar evolution do not founder on the shoals of turbulence. Carbon burning may well propagate by turbulent interchange but one is left with the untractable problem of calculating a complex time-dependent hydrodynamical phenomenon. The difficulties are made worse by the complex convectively driven Urca process which may modify significantly the energetics and composition in the brief convective phase that falls between ignition and thermal runaway. The outcome of this process, first suggested by Bohdan Paczynski and calculated in great detail by Icko Iben, is still not clearly understood.

In addition to these difficulties of principle, there was growing circumstantial evidence that 4–8  $M_{\odot}$  stars lose their envelopes to leave stable, cooling, white dwarfs and hence do not even evolve to the condition of carbon ignition. Uncertainty about whether and how to proceed and burgeoning interest in the application of fascinating new physics to the gravitational collapse problem led supernova theorists away from the exploration of degenerate ignition.

The subject returned to the forefront through a conceptual breakthrough in the understanding of type I supernovae. Work by several people on light curves and by Timothy Axelrod on the spectral synthesis of the late-time nebular spectra gave fascinating new evidence that the famous

exponential decay in the light curves of type I supernovae is powered by the radioactive decay of  $^{56}\text{Ni}$ . Nickel is the natural product of degenerate carbon burning.

A model in which the inner portion of a degenerate carbon core is incinerated to nickel, resulting in the total disruption of the star, soon gained popularity. The picture is just that provided by the tentative models of subsonic turbulent carbon burning of the mid 1970s, as pointed out by Ken'ichi Nomoto and others. A crucial underpinning of this class of model is the assumption that supernova distances accord with values of the Hubble constant,  $H_0 \sim 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ . If it should be the case, as some evidence indicates, that  $H_0 \sim 100 \text{ km s}^{-1} \text{ Mpc}$ , the implied lower luminosity requires very little nickel; and carbon burning to produce that small quantity could not account for the observed velocities of type I events. A completely different class of model, perhaps involving gravitational collapse, would then be required.

Nevertheless, the desire to improve our understanding of degenerate carbon ignition has been rekindled. A new ingredient to the numerical approach to this class of problem has been added by Robert Deupree. Deupree has introduced two-dimensional hydrodynamical techniques to explore time-dependent non-local convection and has used them to study the degenerate helium flash. Whether even a two-dimensional treatment can adequately represent the complexities of full three-dimensional convection has been seriously questioned but Deupree's work has nevertheless shown qualitative departures from previous work. Even if not final, the calculations leave little reason to trust any older results based on one-dimensional hydrodynamics and time-independent mixing length convection.

Muller and Arnett have used two-dimensional techniques similar to those of Deupree to re-examine the question of degenerate carbon burning. They find a complex combustion process in which highly non-spherical convective tongues of burned material reach out from the central core accompanied by a cascade of eddies to a size limited by the numerical grid. Though the process is more complex, the net result is approximately the same as simple one-dimensional models assuming turbulent exchange on small length scales. About 1  $M_{\odot}$  of nickel is produced and the star is totally disrupted — an outcome quite consistent with the observations of type I supernovae if  $H_0 \sim 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ .

The calculations of Muller and Arnett are still limited by grid size and a simplified treatment of strong interactions. They are the first of a new breed, however, and show the way to a new era of study of degenerate carbon burning. □

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## Oxford commemoration

# Liquid helium at the Clarendon Laboratory

from N. Kurti

THE Clarendon Laboratory of Oxford has been celebrating the half-century of its entry into 'big league' low-temperature physics. At a ceremony a few weeks ago, David Shoenberg, the last director of the rival Mond Laboratory at Cambridge, dedicated a plaque commemorating the first liquefaction of liquid helium by K. Mendelssohn at Oxford on 13 January 1933. Because the old Clarendon building where the work was done has long since been dismantled, the plaque is fixed to a wall in the geology building at a spot not very far from where the original experiment was carried out.

The first account of this work appeared in *Nature* on 11 February 1933 in a paper by F. Lindemann and T. Keeley entitled 'Helium liquefaction plant at the Clarendon Laboratory, Oxford'. This was an event of some importance on two counts. It announced the first liquefaction of helium in Britain, which thus became the fifth country where experiments down to about 1K could be carried out — the others being The Netherlands, Canada, FRG and the United States. And more important, it was a major step in Lindemann's efforts to bring the Clarendon Laboratory out of the torpor into which it had sunk under R. Clifton's professorship between 1865 and 1916.

Having worked with Nernst before 1914, Lindemann regarded low-temperature physics as a key research topic for the Clarendon. With this in mind, he installed, in 1930, a hydrogen liquefier of F. Simon's design, and when, a couple of years later, Simon conceived and developed the single-expansion 'Simple Simon' helium liquefier, Lindemann saw his chance of beating Cambridge in the cryogenic game and purchased a liquefier. It was brought over by Mendelssohn and within a week of its arrival there was liquid helium in the Clarendon Laboratory.

The paper emphasizes, without explicitly alluding to the grander installation taking shape in Cambridge, the low cost of the Oxford equipment (£350 for the hydrogen liquefaction plant, £30 for the helium liquefier) and points to the use of glass Dewar vessels for liquid hydrogen, since their 'efficiency equalled that claimed for the more complicated double vessels developed by Professor Kapitza'. It is open to conjecture whether it was by design or by chance that the same issue of *Nature* contained a report of the official opening in Cambridge of the Royal Society Mond Laboratory — a fine and spacious building with a helium liquefier still under construction — an article for which the anonymous

author, J. Cockcroft, was paid £3.

Rivalry between Oxford and Cambridge has persisted. At the dedication ceremony, Shoenberg described how, soon after the discovery in the USA of the 'isotope effect' in superconductors, Harwell provided both laboratories with samples of tin isotopes. It was agreed that Cambridge would make magnetic and Oxford electrical measurements. Shoenberg explained that when the Cambridge work was complete, he suggested to Mendelssohn that the two sets of data should be published simultaneously, and, indeed, an Oxford paper was ready within a week. Only later did it emerge that the Oxford work had not even begun when Shoenberg said he was ready to publish.

Shoenberg also recalled Mendelssohn's capacity to make a virtue of necessity in using the small-scale technique which relied on liquid helium made in the measuring apparatus itself. Not having to transfer liquid

helium from one container to another, he kept his apparatus free from contamination and thus avoided the strange and erratic results his colleagues in Cambridge and elsewhere found when studying the properties of the superfluid helium film.

Nostalgia is unavoidable on these occasions. Shoenberg spoke fondly of "those far-off days when the low-temperature physics community was still small and we all knew each other". Rivalry apart, "the Cambridge groups, and I personally, owed a lot to Francis Simon for his support and encouragement of what I might describe as our fledgling efforts at a time when we were in a sense orphaned by Kapitza's abrupt disappearance to Moscow in 1934". But "those good old days are no more", and in Shoenberg's view, a few years after Sam Collins had developed a commercially available liquefier, "low-temperature physics became more and more a bandwagon". But he thought it was right "that we should remember the pioneers, in particular Kurt Mendelssohn and his historic visit to Oxford 50 years ago when, by making the first liquid helium in England, he inaugurated a new and fruitful area in British physics." □

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## Cranial morphology

# A shaky foundation in the structure of the skull?

from R. Presley

IT has become fashionable to regard cranial morphology as a worked-out biological discipline, summarized in monumental works<sup>1,2</sup>. In Europe new theories of the evolution of the vertebrate skull do continue to emerge<sup>3</sup>, but in the English-speaking world the century-old analysis deriving from T. H. Huxley<sup>4</sup> is thought to present a reasonably secure evolutionary story.

Paramount is the ability to recognize 'homologous' structures in different animals, using topographical anatomy, identity of position in serially repeating structures, correspondence of developmental processes and the demonstration in the fossil record of equivalent structures which make phylogenetic sense. Of these methods the anatomical and the developmental are the most testable, and in the case of the vertebrate basicranium they have stood together well, up to now. Thus the canon holds that cartilage precursors of bone show a consistent pattern, the axial trabecular and parachordal system having a pair of cartilage wings, the orbitosphenoids, whose replacement bone separates cranial cavity from orbit. Much modern experimental science rests on the ability to recognize homologous parts,

modified by treatment, by the canonical criteria and thus to infer the affected organogenetic processes. More generally, recognizing apparently similar phenotypes, we deduce a relationship of genotype.

The findings of Bellairs and Gans published in this issue of *Nature* (p.243) strike a great blow at this edifice. Amphisbaenians, a little-known group of burrowing reptiles usually written-off as modified lizards, have an orbitosphenoid arising directly from membrane bone. So like the classical cartilage bone is it that it has previously<sup>5</sup> been so assigned. Membrane bone can spread along many connective-tissue planes and take on a great variety of forms<sup>6</sup>. It is easy, indeed necessary, in evolutionary theory to admit of major transformations of membrane bones, but the cartilage bones have seemed much more conservative. With the discovery that membrane bone can mimic totally a basic cartilaginous constituent of the developing skull, we must now ask how widespread such a phenomenon may have been in vertebrate evolution. We cannot know the developmental histology of fossils. May we now assume that any problematical elements in their skulls could be *ersatz*





The amphisbaenian Weigmann's Burrowing Lizard (*Trogonophis weigmanni*). The structure of its skull would have rivetted the anatomical and zoological world of the 1920s. Will it do so today?

membrane bone specializations?

As herpetologists, Bellairs and Gans address the taxonomic problem posed by this unique feature of amphisbaenians. Clearly the group can only with difficulty now be dismissed as aberrant close relatives of lizards (cladists should be particularly engaged by this synapomorphy, not known in other tetrapods). There is some comfort for the classical view: a 'vestige' of cartilage is present and much of the wing in some microteiidids may be formed as membrane bone extensions from a more central cartilage anlage. The change may therefore be explicable, but it must have arisen in the fossil record. Where? Has this sort of element been invented by other, now extinct, groups and escaped our notice because of the prophetic powers of morphological canon?

More fundamentally, this bone is not an 'orbitosphenoid' by developmental criteria though it is by anatomy. By not producing cartilage, its cells are not transcribing a normally expressed part of the vertebrate genome. A prop of the homological method has been substantially weakened. Can any experimental embryologists or teratologist now with confidence recognize homologues in treated animals by anatomical or histological criteria? In the 'field-gradient' interpretation of the results of transplantation, are we looking at genome-based homologues or at *de novo* responses to mechanical and functional stresses independent of any morphotype? The paper of Bellairs and Gans would have rivetted the anatomical and zoological world of the 1920s. It should do so today. □

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## Cytoskeleton components

# A comprehensive catalogue of cytokeratins

from Brian Anderton

CELL biologists with an interest in the cytoskeleton can at last safely feel that the inventory of intermediate filament structural proteins is more or less complete. Largely through the monumental efforts of Werner Franke and his colleagues at the German Cancer Research Centre a catalogue<sup>1</sup> of human cytokeratins has now appeared — nineteen different cytokeratin polypeptides in all and each probably a unique gene product.

This is quite remarkable when one considers that the four other members of the intermediate filament class of fibrous organelles have a much more restricted composition: vimentin (one gene expressed in merenchymal cells), desmin (one polypeptide in myogenic cells), glial fibrillary acidic protein (one polypeptide in astrocytes) and neurofilaments (three polypeptides in neurones).

Franke's group has assembled its data by meticulously analysing two-dimensional electrophoretograms of cytoskeletons (enriched in intermediate filaments) from many different epithelioid tissues, cell lines and epithelial tumours (carcinomas). Where necessary, pieces of tissue, including tumours, have been microdissected so as to minimize contamination. The cytokeratin polypeptides range in apparent molecular weights from 40,000 to 68,000 and isoelectric *pH* values from 5 to 8, with a preponderance of the larger proteins being basic and the smaller ones acidic.

Complementary to the chemical analysis, monoclonal antibodies to the cytokeratins have also been used in several laboratories to attack the problem of cytokeratin heterogeneity. As might be expected, some antibodies are more specific than others<sup>2-5</sup>.

The immediate benefits of the catalogue are twofold. First, neoplastic cells seem to express quite faithfully the intermediate filament type of the parent cell; antibodies to the different intermediate filament classes are already being used for tumour diagnosis<sup>6,7</sup>, with commercial exploitation in the pipeline. Monoclonal antibodies to the individual cytokeratins and at least those capable of discriminating between simple and stratified squamous epithelia<sup>2,5</sup> will further refine diagnosis. Second, intermediate filaments appear at appropriate stages and places during embryogenesis and the complexity of the cytokeratins is likely to make them useful as differentiation markers for embryologists<sup>8,9</sup>.

There remains the question, however, of whether or not cells in culture change their pattern of intermediate filament expression. Many feel that tissue culture of

epithelial cells brings about vimentin expression and cytokeratins only would be found in the normal or even neoplastic parent cell.

Franke and his colleagues<sup>10</sup> have also just reported some interesting findings relevant to this problem. They find that a bovine mammary epithelium cell line cultured in the presence of the hormones hydrocortisone, insulin and prolactin expresses cytokeratins typical of the normal tissue cells and contains no vimentin, but a similar cell line propagated without hormone supplement contains vimentin as well as a cytokeratin pattern reminiscent of that of another bovine epithelial cell line which had been maintained in culture for a long time. This suggests that the pattern of intermediate filament expression may be influenced by the cell's environment. This suggestion is borne out to some extent *in vivo* by the discovery by Frans Ramaeckers and colleagues that metastasizing carcinoma cells in the pleural fluids express vimentin, whereas the primary and solid secondary tumours contain cytokeratin but no vimentin<sup>11</sup>.

So we are still probably no nearer to knowing what intermediate filaments do in cells and this, after all, is the *raison d'être* for studying them. The efforts of cell biologists in this field have, however, provided some very useful knowledge for embryologists and pathologists. Clues to function may perhaps also come from paying more attention to finding conditions which modulate intermediate filament expression rather than rigidly searching for properties of cells dependent on the complement of intermediate filament proteins. □

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## Structural chemistry

## Complete structure of palytoxin elucidated

from Yuzuru Shimizu

ABOUT two years ago, two groups — one at Hawaii led by R. Moore and the other at Nagoya, Japan, led by Y. Hirata — independently reported the elucidation of the gross structure of palytoxin, a potent neurotoxin of marine origin<sup>1,2</sup>. The news created a considerable sensation among chemists and toxicologists. The toxin, with a molecular formula  $C_{129}H_{223}N_3O_{54}$  (molecular weight 2,678.5), has no repeating unit and is frilled with many functional groups, including hydroxyl groups, and is considered to be the most complicated molecule ever elucidated by the conventional method without the use of X-ray crystallography. At the time, however, few people imagined that the stereochemistry of the whole molecule, which includes 64 chiral centres and seven double bonds allowing geometrical isomerism, could ever be solved in the near future. In the 15 December issue of *Journal of the American Chemical Society*, four consecutive communications appeared as joint contributions from a group at Harvard headed by Y. Kishi and the aforementioned Japanese group<sup>3-6</sup>. In the last of the four communications, the authors present the structure of palytoxin with complete stereochemistry including absolute configurations.

The publication had, in fact, been preceded by another report on the stereochemical structure of palytoxin from the Hawaii group<sup>7</sup>. The two structures differed in the assignment of 11 chiral centres in the molecule. The structure presented by Professor Kishi's group does appear to be unequivocal, however, as it is supported by synthetic evidence.

Palytoxin was first isolated by J. Scheuer's group at the University of Hawaii from a marine zoanthid, *Palythoa toxica*<sup>8</sup>, and later from other *Palythoa* species — there are minor structural variations depending on the sources and locations. For their research the Hawaii group used mostly the toxin from one location at Maui Island, Hawaii, and the Japanese group, material collected from Okinawa. The toxin is the most deadly nonproteinous one ever isolated and its reported LD<sub>50</sub> of 0.15 µg per kg (intravenous injection in mice) makes it 50 times more toxic than tetrodotoxin and saxitoxin. Its mechanism of action, which seems rather complex, has raised tremendous interest among pharmacologists and it is also known to exhibit strong cytotoxicity against malignant cells.

The toxin consists of a 115-carbon straight-chain backbone with several cyclic

ethers, methyl groups and numerous hydroxyl groups. The ends of the chain are linked with a primary amino group and a small amide-containing moiety. The gross structure was elucidated by both the Hawaii and Japanese groups by first establishing the structures of various fragments and later their connectivity.

Kishi tackled the stereochemistry by synthesizing all the essential fragments derived from the oxidative cleavage of palytoxin in strictly stereochemically controlled conditions, and matching them with samples obtained from the natural compound. This approach, which obviously aims at the total synthesis of the toxin and requires knowledge of the stereochemistry of the molecule, was very clever in running the synthetic work and the stereochemical determination together. The astonishing thing is that despite the complex stereochemistry the Harvard group synthesized all the fragments in a very short time — less than two years.

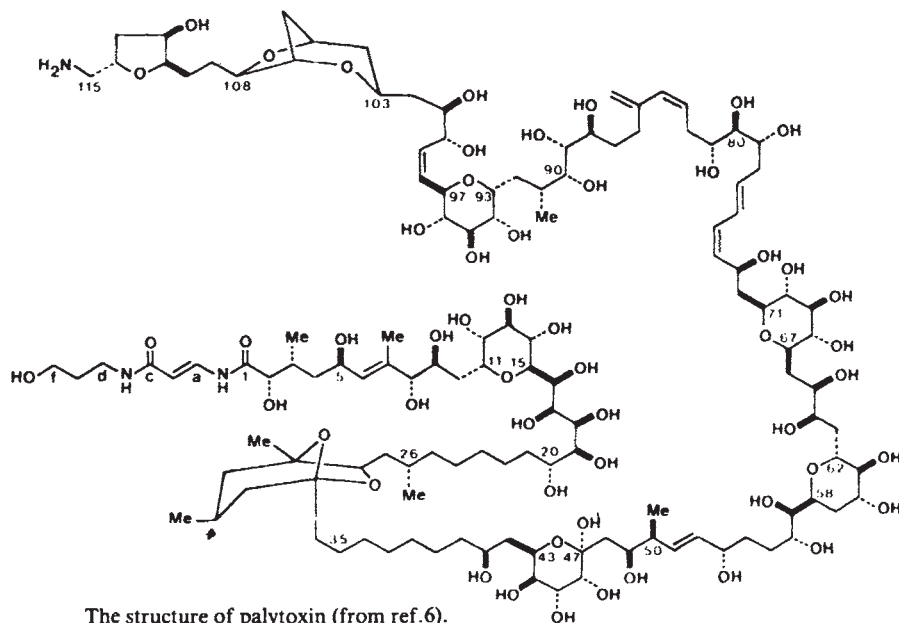
Behind this breakthrough are the recent rapid developments in the stereospecific synthesis of polyhydroxylated natural products. Beginning with the synthesis of macrocyclic antibiotics, and then of polyalkylether-type antibiotics, in which Kishi's group was notably successful, synthetic chemists are now finding ways to synthesize stereospecifically various pentoses, hexoses and amino sugars, which no synthetic chemist dared to undertake decades ago. With the methodology at hand, it is natural for synthetic chemists to look at palytoxin with its numerous chiral centres and drastic biological activity as a challenging molecule. At least one more group is known to be competing with the

Harvard group to totally synthesize palytoxin<sup>9</sup>.

The Hawaii group's approach relied heavily upon high-resolution NMR spectroscopy. They carried out literally thousands of laborious proton-proton spin-decoupling studies and, although the results may have led to some erroneous conclusions, the effort was well worthwhile as a test case to determine the limitations of the approach in structural determination. The failure to assign several chiral centres derives partly from the dilemma intrinsic to the approach — to predict the stereochemistry of certain chiral centres from the spin-spin coupling data in NMR, one has to assume a certain conformation or shape of the molecule at the measurement. It is not an easy task to predict conformation, especially with such a large flexible molecule as palytoxin. Normally the conformation of a molecule is deduced from the NMR data of nuclei with known stereochemistry.

At all events, this seems to be a triumphant moment for synthetic chemists. The Harvard group concluded their report by saying "(the misassignment by NMR methods) points out the limitation of these types of experiments and the continuing importance of organic synthesis in structure elucidation". □

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The structure of palytoxin (from ref. 6).

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## Meteoritics

# Isotopic anomalies in meteorites explained?

from J. H. Reynolds

TWO young chemists at the University of California at San Diego have just reported in *Science* (219, 1073; 1983) a novel isotopic effect. The work by M. H. Thiemens and J. E. Heidenreich III may provide a simple 'chemical' explanation for isotopic anomalies in meteorites, discovered a decade ago by R. Clayton, L. Grossman and T. Mayeda of the University of Chicago. The anomalies have been widely attributed to a nuclear process and have focused much attention on the possible roles played by supernovae in the early history of the Solar System. The new explanation proposed for these effects is so simple that meteorite cosmochemists might well be embarrassed by its having been overlooked.

Mass spectroscopists express isotopic anomalies in ' $\delta$  values'. The  $\delta$  value for an isotopic ratio is simply the fractional difference between the ratio and a standard value for that ratio, expressed in per cent, per mil, or even parts per 10,000 if the effect is very small.

In oxygen,  $\delta 17$  and  $\delta 18$  refer to the ratios  $^{17}\text{O}/^{16}\text{O}$  and  $^{18}\text{O}/^{16}\text{O}$  respectively. For oxygen from terrestrial samples the measured  $\delta$  values almost invariably plot on a line of slope 1/2 when  $\delta 17$  is the ordinate and  $\delta 18$  is the abscissa. This behaviour corresponds to 'mass-dependent fractionation' and means that the terrestrial processes for isotope separation in oxygen are twice as effective when the mass difference involved is twice as great.

Clayton *et al.*'s discovery in 1973 that oxygen samples from carbonaceous meteorites define a line of slope 1 instead of slope 1/2 was an outstanding one for meteoritics. Using the effect, Clayton has been able, for example, to classify Solar System material according to its isotopic patterns for oxygen, an achievement which should eventually be an important boundary condition in discussing the origin of the Solar System. Because the chemical processes which operate terrestrially on oxygen have always produced samples on the line of slope 1/2, the meteoritic effect has been attributed to a nuclear process.

The experiment by Thiemens and Heidenreich is elegantly simple. They subject oxygen gas at reduced pressure to a Tesla discharge in a vessel which is partially immersed in liquid nitrogen. The ozone produced in the discharge freezes out on the cooled walls, so that after a time the residual oxygen can be pumped away and collected and the condensed ozone then warmed up and collected as a second fraction.

The isotopic compositions of the oxygen

in the complementary samples have been measured in two different laboratories and show complementary displacements from the starting material precisely on the line of slope 1. The extent of the separation depends on how long the discharge is applied, with  $\delta$  values of up to 8 per cent having been observed. The experiment immediately establishes that nuclear processes need not necessarily be invoked to account for isotopic compositions in oxygen which are displaced from normal on a line of slope 1.

## Royal Greenwich Observatory

# Satellite laser ranging in the UK

from A. J. Meadows

THE new satellite laser-ranging facility at the Royal Greenwich Observatory is nearing completion and is expected to obtain usable data by April this year and fully calibrated data by the autumn. A workshop was held at the Observatory last month to discuss plans for the laser and to examine how ranging data should be processed, analysed and exploited in the UK.

Satellite laser ranging developed rapidly during the 1970s as a method of determining the distance of satellites from observing stations on the Earth's surface with high accuracy. The resultant data not only provide a better picture of the forces acting on satellites, but they can also be used to fix very precisely the position and motion of the observing station. Much of the work in this area has been carried out in the US, with NASA playing a leading part, but several European countries have also participated. The UK has so far been conspicuous by its absence.

For most applications of satellite laser-ranging data, it is necessary to combine information from more than one station. International cooperation is therefore essential, and an agreement has already been reached between SERC and NASA for the exchange of data. However, the UK results will not simply extend the existing number of observations. The laser facility to be installed at the Royal Greenwich Observatory is one of the first third-generation systems to come into operation and should ultimately be capable of measuring distances to satellites with errors of a centimetre or so. This is nearly an order of magnitude improvement over older systems and increases the likelihood that a number of effects of geophysical interest, including continental drift, will be

Thiemens and Heidenreich think it most likely that a shielding effect is operative in a photochemical step in their reaction. If the Tesla discharge produces a band of 'white' ultraviolet radiation which is responsible for dissociating the oxygen molecules and initiating the reaction which leads to ozone, the photons which liberate  $^{17}\text{O}$  are predominantly those tuned to dissociating the  $^{17}\text{O}^{16}\text{O}$  molecule and are distinct in wavelength from those tuned to the  $^{16}\text{O}_2$  molecule. In a one-dimensional geometry where the photons travel from a source plane through a slab of gas of length  $L$ , the initial dissociation ratio for those two types of molecule (labelled 2 and 1 respectively) can easily be shown to be:

$$Q_2/Q_1 = [1 - \exp(-N_2\sigma_2L)] / [1 - \exp(-N_1\sigma_1L)]$$

with a similar expression for the initial dissociation ratio  $Q_3/Q_1$  where the subscript 3 refers to the  $^{18}\text{O}^{16}\text{O}$  molecule.

detected. At the moment UK observing possibilities are limited to ten satellites, as only satellites fitted with retro-reflectors can be ranged by laser. Retro-reflectors are, fortunately, very easy to fit to satellites so the number is expected to increase considerably over the next decade.

Two areas of particular interest were identified. First, the facility should be ready in time to allow participation in the international MERIT campaign, aimed to provide better measurements of Earth rotation, from September 1983 to October 1984. Second, laser ranging to satellites equipped with altimetry instruments may reduce uncertainties in defining their orbits, so improving observations of land and sea profiles around the UK.

Laser-ranging measurements are relevant to much geophysical and geodetic research and it was agreed that all interested workers should be alerted to the proposals emanating from the workshop. It was also thought that it would be valuable to obtain feedback on possible longer-term developments — should, for example, an attempt be made to link the stationary laser facility at the Royal Greenwich Observatory to a mobile laser in order to increase the scope of UK geophysical and geodetic measurements? One immediate result of the workshop will therefore be a general meeting, to be held later this year, where information on the possibilities of the satellite laser-ranging facility will be presented to the widest possible audience. □

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The quantities  $N$  and  $\sigma$  refer to the number of molecules per unit volume and their cross-section for absorbing the disruptive radiation respectively. The cross-sections  $\sigma_1$ ,  $\sigma_2$  and  $\sigma_3$  will be equal in the conditions we have specified. As both  $^{17}\text{O}$  and  $^{18}\text{O}$  are very rare species compared with  $^{16}\text{O}$ , the exponentials in the numerators of both dissociation ratios can be closely approximated by the expression  $1 - N\sigma L$ .

The consequence is that the initial dissociation ratio  $Q_2/Q_3$  is just  $N_2/N_3$  and the initial production rates of ozone molecules containing  $^{17}\text{O}$  and  $^{18}\text{O}$  will be nearly proportional to the amounts of those isotopes originally present, whereas the abundance in ozone of  $^{16}\text{O}$  will be relatively less because of the greater shielding by the species  $^{16}\text{O}_2$ . The shielding thus produces samples on the line of slope 1 as observed.

The essential condition for observing this kind of fractionation, which oxygen well fulfills, is to have two or more isotopes of low abundance. The effect could be quite a general one in oxygen, taking place for several reactions induced photochemically on different oxygen-bearing reactants. Nor would  $P$  and  $T$  be much constrained for these processes to occur.

Thiemens and Hidenreich's researches are still in their infancy, but already the prognosis for establishing the shielding process as the relevant mechanism in the laboratory experiments is quite good. Since their first report, they have already observed similar results for  $\text{CO}_2$ , as would be predicted.

Whether the effect has been cosmochemically significant will be more difficult to establish. Attributing the effects seen in meteorites to the photochemical process has advantages. If the effect in meteorites is nuclear, the high chemical abundance of oxygen requires that much more isotopically anomalous material would have existed in the early Solar System than other isotopic anomalies seen in meteorites require.

Also, the question of why silicon does not show similar isotopic anomalies would be answered: in silicon, the two isotopes present in low abundance are 44 times more abundant on the average than their oxygen counterparts. And finally, it has always been a little peculiar that the oxygen effect should have a slope just equal to 1 when various slopes could result in mixing oxygen from different nucleosynthetic pots.

It is certainly too early to tell, but it may very well be that the Clayton effect will eventually be interpreted in terms of early electromagnetic radiation in the Solar System and not in terms of the distribution of supernova debris. □

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## International lithosphere programme

# Deep seismic profiling plans in Australia

from M.W. McElhinny

THE International Lithosphere Program (ILP) in Australia got off to an enthusiastic start at the Sixth Convention of the Geological Society of Australia held in Canberra from 21 to 25 February. The Australian National Committee for the ILP sponsored four symposia on the areas most appropriate in the Program's initial stages: lithospheric transect studies of the Australian continent (LITSAC); recent plate movements and deformations in the Australasian region; oceanic lithosphere studies around Australia; and intraplate igneous activity in Australasia.

LITSAC (chairman R. Rutland) is the main programme. Committees will be set up in each state to coordinate the choice of sites for major transect studies; these will form part of the Australian Continental Reflection Profiling Program (ACORP) — itself based on the highly successful COCORP project in North America. Unlike COCORP which used the Vibroseis method, however, the ACORP programme will use explosion seismology; Australia is less densely populated and environmental constraints are not so severe.

The first major study, involving nearly 1,500 km of deep seismic profiling of the Central Eromanga Basin in western Queensland, has already been carried out by the Bureau of Mineral Resources. Reflections were obtained from between 20 and 60 km depth underneath a seismically homogeneous upper crust lying at between 6 and 20 km depth (D. Finlayson and S. Mathur). The technical standard of the sections and the clarity of the deep reflections were particularly impressive and clearly showed that explosion seismology could have advantages over the Vibroseis technique in deep reflection studies.

Proposals for future deep seismic reflection profiles were put forward for New South Wales, Tasmania, Western Australia and Queensland.

It was emphasized that to attract financial support from a wide range of funding agencies, the initial profiles should yield results that can readily be interpreted in terms of known upper crustal geology, make important contributions to the understanding of major regional problems, are of general significance in terms of crustal evolution and have some implications for broad-scale resource assessment.

To satisfy these conditions the New South Wales LITSAC group proposed a traverse in eastern Australia from the eastern margin of the Lachlan Fold Belt across the Gunnedah Basin and the southern part of the New England Fold

Belt. Contact between the Lachlan and New England Fold Belts is nowhere exposed on the surface but must occur along the profile. Information on the Lachlan-New England relationship will be important for tectonic and structural interpretations. The New England section is presumed to be representative of an arc-forearc basin-subduction complex scheme, and may be one of the best and most complete examples of an accretionary convergent margin preserved in the geological record. The deep structure of these margins and their evolution as they are incorporated into continental crust are little understood. The profile would contribute much to our knowledge of these processes and thus of Phanerozoic continental growth in general. Deep crustal structures of a very general kind were obtained by the LISPB programme across the Caledonian system in Britain, but otherwise deep-crustal profiling of any detail is lacking across cratonized arc-forearc terranes.

In complete contrast, M. Bickle and R. Gee proposed a series of carefully selected seismic reflection profiles in the West Australian Shield which, when integrated with other data, could provide information on several critical issues concerning early crustal evolution and structure. Of particular importance was the possibility of determining the three-dimensional shape of greenstone belts — one of the outstanding enigmas of Archaean geology. The most puzzling feature of the West Australian Shield is the deep crustal nature of the southern margin of the buried Pilbara Craton. The evolution of Archaean nuclei in Precambrian shields is still a major problem to be solved and it is evident that the Yilgarn and Pilbara Cratons, and the intervening region, had their own characteristic crustal features. A 200 km traverse across the southern margin of the Pilbara Craton could thus be particularly valuable.

The symposium demonstrated the enthusiasm in the Australian geoscience community for the major multidisciplinary programmes based around LITSAC and deep-seismic profiling. The fact that the ILP has been a catalyst in formulating a nationwide programme in Australia must be regarded as one of its major initial successes. □

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# Cosmological implications of helium and deuterium abundances on Jupiter and Saturn

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*The determination of relative abundances of helium and deuterium in Jupiter and Saturn provides estimates of the primordial abundances of these elements. The values so inferred can be compared with theoretical predictions of the standard model of the big-bang theory as a test of the model and suggest either that deuterium is more efficiently consumed in subsequent nucleosynthesis than hitherto assumed, or that the standard big-bang model should be revised in order for there to have been less production of primordial helium.*

THE standard big-bang theory predicts that the relative amounts of primordial helium and deuterium depend on the ratio of baryons to photons in the Universe. The greater the number of baryons, the higher the abundance of helium and the lower the amount of deuterium. A determination of these primordial abundances therefore allows one to assess whether or not there is sufficient matter to close the Universe, provided neutrinos have no mass. Such a determination also permits an evaluation of the big-bang theory itself, by testing whether the primordial abundances of helium and deuterium fit the predictions of the theory. The problem in making these tests is to find an undisturbed reservoir of hydrogen, helium, and deuterium in which the relative abundances can be determined. We believe that the atmosphere of Jupiter provides just such a reservoir.

Owing to the large masses and low-exospheric temperatures of the giant planets, even the lightest elements have not escaped from their atmospheres since these planets formed. The helium in Jupiter must, therefore, be composed of a primordial component produced in the big bang and of a minor component subsequently produced in stars and injected into the interstellar medium by explosions of supernovae and by stellar winds. The measurement of the jovian helium abundance then provides an upper limit to the primordial helium abundance.

Deuterium is also produced in the big-bang but is destroyed in stars. As a consequence, the D/H ratio in the interstellar medium has decreased as a function of the time since the origin of the Universe. The measurement of this ratio in Jupiter's atmosphere provides the interstellar value of D/H 4,550 Myr ago, while measurements in the interstellar medium provide current values of D/H in various regions of our Galaxy. The jovian value is then a lower limit on the primordial deuterium abundance, which should be higher than local interstellar values. Given the jovian and interstellar abundances, models of chemical evolution in the galaxy provide estimates of the primordial values of D/H and He/H. We will compare these derived primordial abundances to values predicted by the standard model of the big-bang theory.

## Helium and deuterium in Jupiter and Saturn

The helium abundance in Jupiter has been determined from Voyager data by two methods<sup>1</sup>. One method takes advantage of the different spectral dependences of the absorption coefficients corresponding to H<sub>2</sub>-H<sub>2</sub> and H<sub>2</sub>-He collisions in the 300-700 cm<sup>-1</sup> region<sup>2</sup>. The hydrogen-to-helium mixing ratio can be retrieved from many different areas on Jupiter so

that uncertainties due to random errors become negligible. From an average of 62 different retrievals, the inferred value of the hydrogen mole fraction is  $q = 0.897 \pm 0.03$ . Here  $q$  is defined as

$$q = \frac{n_{\text{H}_2}}{n_{\text{H}_2} + n_{\text{He}}}$$

where  $n$  is the number density of the indicated gas. The main cause of the uncertainty in this determination comes from the uncertainty in the absorption coefficients. Neglecting the small contribution of heavy elements, the helium mass fraction is approximately:

$$Y = \frac{1-q}{1-0.5q}$$

The derived helium mass fraction for Jupiter is then  $Y = 0.19 \pm 0.05$ .

The second method for determining the helium abundance uses a thermal profile independently derived from radio occultation measurements. The temperatures and pressures determined in this way are proportional to the mean molecular weight and thus to the hydrogen-to-helium ratio. This method is very sensitive to the helium abundance and depends only slightly on the accuracy of the H<sub>2</sub>-H<sub>2</sub> and H<sub>2</sub>-He absorption coefficients. However, it strongly depends on the accuracy of the retrieved thermal profile (for a given value of the mean molecular weight) and is limited to two results for each flyby encounter (ingress and egress occultations).

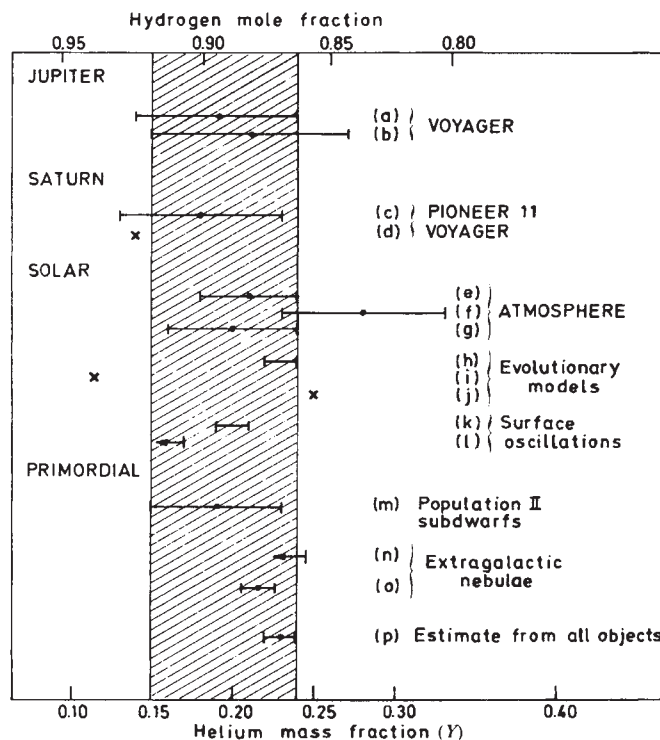
This method gives  $q = 0.88 \pm 0.036$ , that is  $Y = 0.21 \pm 0.06$ .

Combining the values obtained by the two methods we obtain:

$$0.845 < q < 0.915$$

$$0.15 < Y < 0.24$$

The crucial question here is to determine whether or not the helium abundance in the outer atmosphere of Jupiter is representative of the composition of the primitive nebula. Because of the high vapour pressures of hydrogen and helium at low temperatures, these gases were entirely in a gaseous form in the primitive nebula and no fractionation of helium is envisaged in any scenario of the formation of the planet<sup>3</sup>. However, as originally proposed by Smoluchowski<sup>4</sup>, a differentiation of helium from hydrogen could have occurred subsequently as these gases could be immiscible over the range of temperature and pressure appropriate to the interiors of the giant planets<sup>5,6</sup>.



**Fig. 1** Comparison of helium mass determinations in the Solar System with the primordial values derived from other objects. The shaded area corresponds to the  $1\sigma$  error bars on the jovian value derived from Voyager investigations ((a) and (b)). See Table 1 for letters (a)–(p).

**Table 1** Summary of helium abundances

| Determination                                                       | $q$                   | $Y$               | Refs  |
|---------------------------------------------------------------------|-----------------------|-------------------|-------|
| <b>Jupiter</b>                                                      |                       |                   |       |
| (a) Voyager IRIS                                                    | $0.897 \pm 0.03$      | $0.19 \pm 0.05$   |       |
| (b) Voyager IRIS combined with radio occultation                    | $0.880 \pm 0.036$     | $0.21 \pm 0.06$   | 1     |
| (a) + (b)                                                           | $0.89 \pm 0.027$      | $0.20 \pm 0.04$   |       |
| <b>Saturn</b>                                                       |                       |                   |       |
| (c) Pioneer 11                                                      | $0.90 \pm 0.03$       | $0.18 \pm 0.05$   | 35    |
| (d) Voyager IRIS                                                    | $\sim 0.925$          | $\sim 0.14$       | 7     |
| <b>Solar</b>                                                        |                       |                   |       |
| (e) Helium emission lines                                           | $0.88 \pm 0.02$       | $0.21 \pm 0.03$   | 36    |
| (f) Helium emission lines                                           | $0.84 \pm 0.03$       | $0.28 \pm 0.05$   | 37    |
| (g) Cosmic rays                                                     |                       | $0.20 \pm 0.04$   | 38    |
| (h) Standard interior models (initial abundance)                    | $0.86 - 0.88$         | $0.22 - 0.24$     | 39–43 |
| (i) Low heavy element fit                                           | 0.939                 | 0.115             | 40    |
| (j) Well mixed interior                                             | 0.86                  | 0.25              | 44    |
| (k) Solar oscillations                                              | $0.88 - 0.90$         | $0.19 - 0.21$     | 14    |
| (l) Solar oscillations                                              | $> 0.91$              | $< 0.17$          | 15    |
| <b>Primordial</b>                                                   |                       |                   |       |
| (m) Population subdwarf                                             | $0.90 \pm 0.02$       | $0.19 \pm 0.04$   | 45    |
| (n) Extragalactic H II emission                                     | $\leq 0.86 \pm 0.002$ | $0.245 \pm 0.003$ | 22    |
| (o) Extragalactic H II emission                                     | $0.88 \pm 0.01$       | $0.216 \pm 0.01$  | 18    |
| (p) Best estimate from all results                                  | $0.87 \pm 0.01$       | $0.23 \pm 0.01$   | 17    |
| Other results from extra-galactic H II emission not shown on Fig. 1 | $0.871 \pm 0.009$     | $0.228 \pm 0.014$ | 19    |
|                                                                     | $0.879 \pm 0.009$     | $0.216 \pm 0.015$ | 20    |
|                                                                     | $0.879 \pm 0.008$     | $0.216 \pm 0.013$ | 21    |

The existence of such a phenomenon is confirmed by the results of the determination of the helium abundance in Saturn by Voyager. Preliminary results<sup>7</sup> indicate that  $Y \approx 0.13 \sim 0.14$  which is significantly less than the jovian and solar values (Table 1 and Fig. 1).

However, considerations based on energetics indicate that helium differentiation has not yet begun in Jupiter. The intensity of the internal energy source has been accurately measured from Voyager by Hanel *et al.*<sup>8</sup>. This value is in a very good agreement with the prediction of the present luminosity of Jupiter from homogeneous evolutionary models which assume no differentiation of helium<sup>9–11</sup>. If differentiation were occurring, the immiscibility of helium would have led to the formation of helium droplets which, migrating towards the centre of the planet, would have liberated gravitational energy resulting in a jovian luminosity more intense than the observed value.

On the other hand, homogeneous evolutionary models for Saturn predict luminosity decay times of only 2,000 Myr (refs 10, 11), so that an additional source of energy is required. The observed depletion of helium in Saturn's atmosphere compared with the solar abundance is sufficient to explain the present luminosity of the planet if differentiation is invoked as the source of this energy flux<sup>12</sup>.

The internal structures of Jupiter and Saturn appear qualitatively different. Helium differentiation has begun in Saturn but not in Jupiter. The atmospheric helium abundance of Jupiter is thus the one corresponding to the abundance in the primitive solar nebula.

The upper limit of the jovian value provides an upper limit of the primordial helium abundance equal to 24% by mass. This value can be compared with cosmic and solar abundances (Table 1 and Fig. 1). As is obvious from the large spread of results for the Sun, a reliable estimate of the solar helium abundance at the time of the Sun's formation remains elusive. The two most recent determinations from helium emission lines are in conflict, although the error bars overlap. Large systematic errors can arise in the interpretation of abundances derived from cosmic rays<sup>13</sup>, resulting in uncertainties far in excess of

the quoted errors. Evolutionary models are able to fit the mass, luminosity and age of the Sun for values of  $Y$  varying from 0.11 to 0.25. The interpretation of surface oscillations made by Iben and Mahaffy<sup>14</sup> and by Isaak<sup>15</sup> is contested by Christensen-Dalsgaard and Gough<sup>16</sup>. As they point out, other measurements are necessary to discriminate safely among various models of the internal structure of the Sun. The jovian determination of helium from Voyager may, in fact, represent the best estimate of the solar value to date<sup>1</sup>.

The determination of the primordial cosmic abundance of helium is also difficult. A review of various techniques used to infer this quantity can be found in ref. 17. Most of these methods are strongly model dependent so that systematic errors are difficult to assess. Accurate estimates were recently proposed from emission line measurements of extragalactic objects<sup>18–21</sup>. These authors measured helium abundances in old galaxies and assumed the validity of the linear relation

$$Y = Y_p + \frac{\Delta Y}{\Delta Z} Z$$

where  $Z$  is the heavy element abundance. Extrapolating the heavy element abundance to zero provides an estimate of the primordial abundance  $Y_p$ . The highest precision announced up to now is from Rayo *et al.*<sup>18</sup> who derive  $Y_p = 0.216 \pm 0.010$  ( $3\sigma$ ) from H II regions of the M101 galaxy.

However, Kunth<sup>22</sup> has contested the validity of the method and claimed that no correlation between  $Y$  and  $Z$  can be established unambiguously so that only an upper limit of  $Y_p$ , as an average of the measurements of  $Y$ , can be inferred. From his analysis of 25 galaxies he derived  $Y_p \leq 0.245 \pm 0.003$ .

On the other hand, the helium abundance derived from NGC5471 by Rayo *et al.*<sup>18</sup> is only  $Y = 0.223 \pm 0.006$  ( $3\sigma$ ), that provides an upper limit of  $Y_p$  significantly lower than the value of Kunth. From a review of all results, Pagel<sup>17</sup> concludes that  $Y_p = 0.23 \pm 0.01$ , which seems reasonable although it is not clear how a lower limit can be established from the available data. For example, in addition to the arguments presented by



Kunth, any determination of the lower limit of  $Y_p$  from observations of galaxies is uncertain as some helium could have been produced as a result of rapid formation of high mass stars before the formation of galaxies (a Population III). This assumption has been invoked by Vigroux<sup>23</sup>, as a possible explanation of the large abundance of iron observed in some clusters of galaxies.

In any case, we re-emphasize that the measurement of the helium abundance in Jupiter's atmosphere provides evidence for a firm upper limit of  $Y_p < 0.24$  that is independent of these arguments. The true value of  $Y_p$  must be less than this because Jupiter's helium includes a stellar component.

The deuterium abundance in the atmosphere of Jupiter was first evaluated by Beer and Taylor<sup>24</sup> from the measurement of absorption lines of  $\text{CH}_3\text{D}$  at  $5\ \mu\text{m}$ . In this method, the derivation of the D/H ratio also requires the determination of the  $\text{CH}_4$  abundance which was controversial before Voyager. The Voyager IR experiment permits a simultaneous determination of  $\text{CH}_4$  and  $\text{CH}_3\text{D}$  mixing ratios from absorption bands in the thermal spectrum of Jupiter. Kunde *et al.*<sup>25</sup> then derived the D/H ratio from:

$$\text{D/H} = \frac{1}{4f} \times \frac{\text{CH}_3\text{D}}{\text{CH}_4}$$

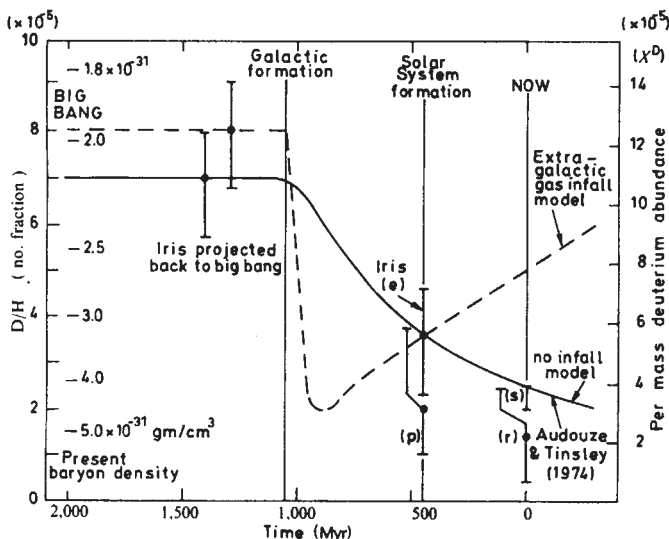
where  $f$  is the fractionation factor estimated to be 1.37 by Beer and Taylor<sup>24</sup> from an analysis of chemical and convective processes in the deep atmosphere of Jupiter. The D/H value in the primitive nebula, 4,550 Myr ago, would then be:

$$\text{D/H} = \left( 3.6^{+1.0}_{-1.4} \right) \times 10^{-5}$$

The per mass abundance for  $Y = 0.23$ , is:

$$X_2(\text{D}) = \left( 5.5^{+1.5}_{-2.1} \right) \times 10^{-5}$$

This value is in reasonable agreement with the estimate of  $2.5 \pm 1.5$  for the protosolar D/H value determined from He in the solar wind using the protosolar  $\text{He}^3/\text{He}^4$  ratio derived from measurements in meteorites<sup>26</sup>.



**Fig. 2** Evolutionary abundance curves for D/H. The D/H ratio by number is on the left scale and the per mass deuterium relative abundance is on the right scale ( $X(\text{D}) = 1.54 \text{ D/H}$  for  $Y = 0.23$ ). The effect of galactic chemical evolution on the D/H ratio is illustrated by two contrasting models of Audouze and Tinsley<sup>28</sup>, normalized to pass through the central jovian D/H value determined from the IRIS Voyager experiment (e)<sup>25</sup>. The determination labelled (p) is derived from solar wind  $\text{He}^3$  measurements by Geiss and Boschler<sup>26</sup>. (r) and (s) refer to interstellar medium measurements from Copernicus by Laurent *et al.*<sup>27</sup> and by Bruston *et al.*<sup>28</sup>. Note that evolutionary models predict a destruction of deuterium  $\leq 3$  between the origin of the Universe and now (adapted from Kunde *et al.*<sup>25</sup>).

The value of D/H in the galaxy now is  $(1.5 \pm 1.0) \times 10^{-5}$  as summarized by Laurent *et al.*<sup>27</sup> from various interstellar medium measurements (that is  $X_2(\text{D}) = (2.3 \pm 1.5) \times 10^{-5}$ ). Bruston *et al.*<sup>28</sup>, from a re-analysis of Copernicus data, had derived  $\text{D/H} = 2.25 \pm 0.25 \times 10^{-5}$ , but from new arguments Vidal-Madjar *et al.*<sup>29</sup> now favour the lowest determinations,  $\sim 0.5 \times 10^{-5}$  ( $0.75 \times 10^{-5}$  by mass).

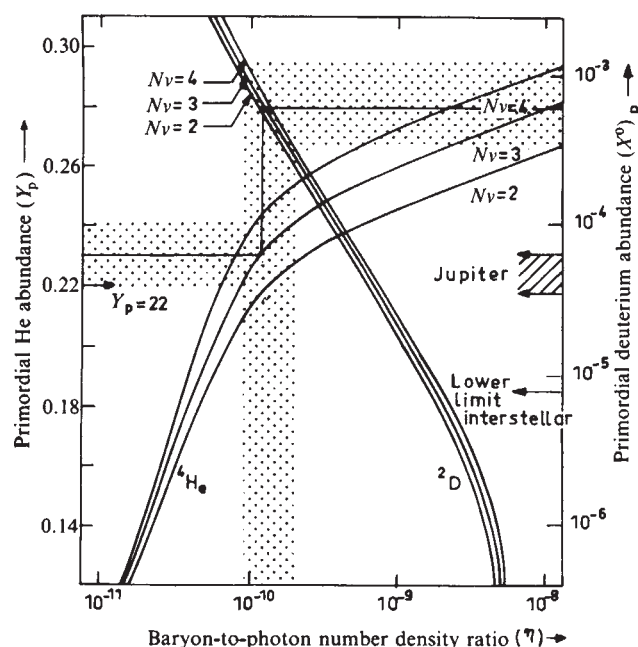
All these values are plotted on Fig. 2, from ref 25, together with curves of the variation of D/H predicted by the models of Audouze and Tinsley<sup>30</sup> for chemical evolution of the galaxy. The present results seem to exclude an infall model in which extragalactic gas of primordial big-bang composition is assumed to enter the galaxy during its evolution. That is certainly the case if the value  $0.5 \times 10^{-5}$  is confirmed in the interstellar medium. Even in their extreme forms, current models<sup>17,30</sup> for nuclear burning of deuterium in stars predict a primordial abundance of deuterium which should not exceed 5 times the value found in the interstellar medium today.

### Derived primordial He and D abundances compared with big-bang theory predictions

In Fig. 3, adapted from ref. 31, the production of primordial helium and deuterium is illustrated according to the standard model of the big-bang, as a function of the baryon-to-photon number density ratio  $\eta$ .  $Y$  and  $X_2(\text{D})$  are calculated from Table 1 of ref. 32, for  $N_\nu = 2, 3$  and 4 neutrino families. There is some experimental evidence for the existence of at least three neutrino families.

Since helium and deuterium are produced at the same time,  $Y$  and  $X_2$  must correspond to the same value of  $\eta$ . The uncertainty in  $Y_p$  ( $0.22 < Y_p < 0.24$ ) leads for  $N_\nu = 3$ , to an uncertainty in  $\eta$ :  $0.35 \times 10^{-11} < \eta < 2 \times 10^{-10}$ . For this uncertainty, the corresponding abundance of primordial deuterium would be  $3.4 \times 10^{-4} < (X_2(\text{D}))_p < 11.6 \times 10^{-4}$  (for  $N_\nu = 3$ ).

The comparison of this result to the upper limit of the jovian determination ( $X_2 \leq 7 \times 10^{-5}$ ) leads to the conclusion that the primordial abundance must have been reduced (destroyed) by a factor 5–16 between the origin of the Universe and the time of the origin of the Solar System 4,550 Myr ago. If we compare



**Fig. 3** Comparison of calculated primordial abundances of helium and deuterium (by mass) from the big-bang theory with experimental determinations of  $Y_p$  and determinations of deuterium in Jupiter from Kunde *et al.*<sup>25</sup>. The lower limit of interstellar medium measurements, now favoured by Vidal-Madjar *et al.*<sup>29</sup>, is also indicated. The dotted area indicates the correspondence between  $Y_p$  ( $0.22 < Y_p < 0.24$ ),  $\eta$  and the theoretical value of  $X(\text{D})$ . See text for discussion (adapted from ref. 31).

this result to the lower limit found in the interstellar medium today the depletion factor is 44–1,500 between the origin of the Universe and now, much higher than the value of  $\leq 5$  predicted by galactic evolutionary models.

Even if we adopt for the primordial abundance the upper limit  $Y_p = 0.24$  and for the jovian deuterium value the upper limit  $7 \times 10^{-5}$ , the resulting depletion factor is larger than those commonly admitted in models of chemical evolution of galaxies. Should future determinations of the helium primordial abundance lead to  $Y_p \leq 0.23$ , as already obtained by Rayo *et al.*<sup>18</sup>, or laboratory experiments in preparation demonstrate that  $N_\nu \geq 4$ , the degree of deuterium depletion inferred from Fig. 3 would be even harder to explain in the framework of present evolution theories which must also account for the observed cosmic abundances of the heavier elements.

In the absence of such an explanation, the standard model of the big-bang theory would have to be revised to produce less helium. Various *ad hoc* modifications of assumptions of the model have already been envisaged as for instance by Rana<sup>31</sup>, who proposes a slight neutrino degeneracy: a small excess of neutrinos over antineutrinos.

Because in the standard model, the helium production depends on the baryon-to-photon ratio  $\eta$ , on the number of neutrino families  $N_\nu$ , and finally on the lifetime of the neutron, a simpler way to reduce the primordial helium abundance is to decrease, as much as permitted by laboratory experiments, the value of the lifetime of the neutron, as analysed by Olive *et al.*<sup>33</sup>. From an analysis of available experimental data, these authors consider the half-life  $\tau_{1/2}$  in the range  $10.13 < \tau_{1/2} < 10.82$  min while until recently the accepted value was  $10.61 \pm$

0.16 min. Adopting the lowest limit of  $\tau_{1/2}$ , which is only marginally possible considering the measurements given by Olive *et al.*<sup>33</sup>, the relation  $0.22 < Y_p < 0.24$  would lead to  $1 \times 10^{-10} < \eta < 3 \times 10^{-10}$ , and consequently to  $1 \times 5 \times 10^{-4} < (X_2(D))_p < 9 \times 10^{-4}$ . The depletion factor is then from 2.1 to 13 between the big-bang and the formation of Jupiter. In such a case  $Y_p = 0.24$  is quite acceptable, but  $Y_p < 0.23$  still results in a deuterium depletion factor higher than 4.5. Note that Schramm<sup>34</sup> adopts for  $\tau_{1/2}$  the more plausible range  $10.4 < \tau_{1/2} < 10.8$  min.

## Conclusions

A large and decisive improvement in the determination of the relative abundance of helium and deuterium has been provided by the Voyager encounters with Jupiter and Saturn. These results lead to the following conclusions: (1) The comparison of the predictions of the standard model of the big-bang theory with observations of helium in Jupiter and in galaxies and observations of deuterium in Jupiter and in the interstellar medium suggests that either much more deuterium was destroyed than is predicted by current theories of chemical evolution of galaxies or the standard model of the big-bang theory must be revised in order to change the proportions in which helium and deuterium are produced.

(2) Unless the standard model is drastically modified, this analysis does not change the present view that the baryon density deduced from current deuterium abundances is insufficient to close the Universe.

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# Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus

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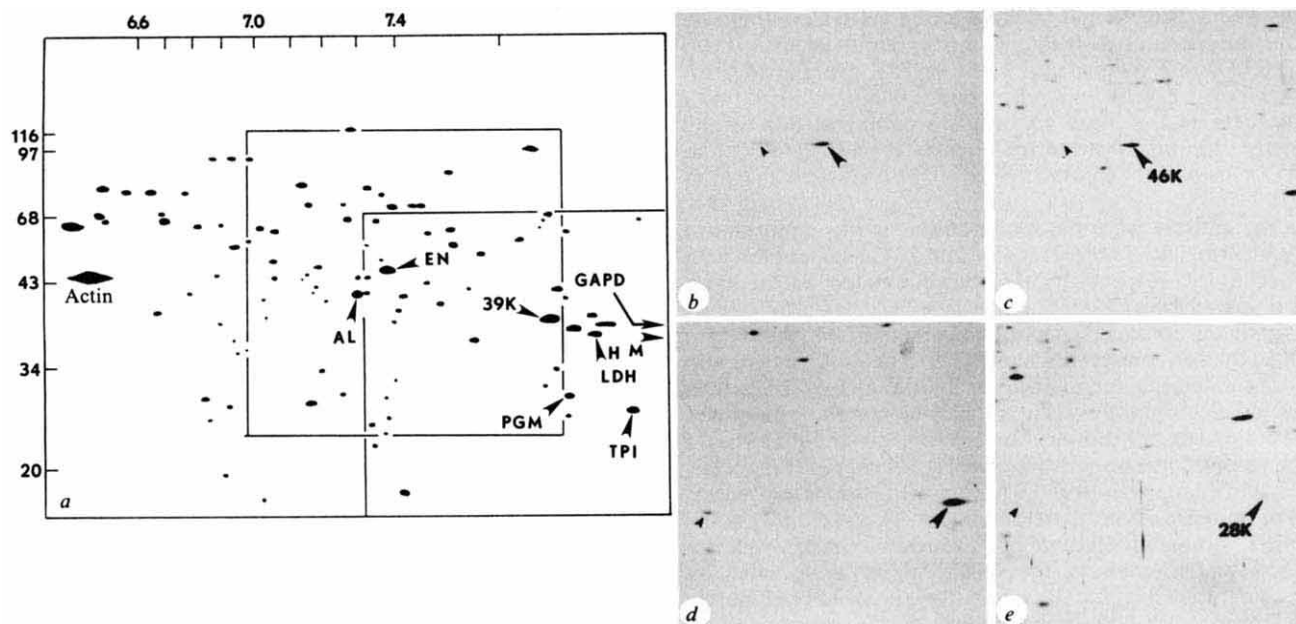
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*Isozymes of enolase, phosphoglycerate mutase and lactate dehydrogenase are phosphorylated at tyrosine in cells transformed by viruses whose transforming proteins are tyrosine protein kinases. The role of these structural modifications in the regulation of glycolysis in such cells is not known.*

PHOSPHORYLATION of cell proteins at tyrosine is rare in most cells in normal conditions of culture. However, fibroblasts transformed by any of several retroviruses, or quiescent fibroblasts briefly exposed to epidermal or platelet-derived growth factor,

have higher than normal levels of phosphotyrosine<sup>1–5</sup>. Because tyrosine-specific protein kinase activities are tightly associated with the relevant virus-transforming proteins and growth factor receptors<sup>1,6–8</sup>, it is presumed that the phosphorylation of cellular





**Fig. 1** Two-dimensional gel analysis of immunoprecipitated chicken fibroblast proteins. *a*, Diagram of silver-stained two-dimensional gel of total chicken fibroblast proteins showing positions of proteins recognized by six different antisera raised to muscle glycolytic enzymes (for abbreviations, see text) and of the 39K ('39K'), 46K ('EN') and 28K ('PGM') tyrosine kinase substrate proteins<sup>18</sup>. GAPD and LDH-M lie off the basic end of the gel at the positions arrowed. Approximate isoelectric points and molecular weights are indicated<sup>11</sup>. PK and PGK were not located. *b*, Part (see left hand box in *a*) of an autoradiograph of a two-dimensional gel loaded with unlabelled total chicken fibroblast proteins mixed with an anti-EN immunoprecipitate of <sup>35</sup>S-methionine-labelled fibroblast proteins. *c*, Staining pattern of the same region of the gel as in *b*. '46K', the 46K tyrosine kinase substrate protein. Small arrowhead, minor protein related to the major 46K protein<sup>18</sup>. *d*, Part (see right hand box in *a*) of an autoradiograph of a two-dimensional gel loaded with unlabelled total chicken fibroblast proteins mixed with an anti-PGM immunoprecipitate of <sup>35</sup>S-methionine-labelled fibroblast proteins. *e*, Staining pattern of the same region of the gel as in *d*. '28K', the 28K tyrosine kinase substrate protein. Small arrowhead, 28K phosphoprotein found in uninfected cells<sup>18</sup>.

**Methods:** Glycolytic enzymes were purified by a procedure to be described in detail elsewhere (N.A.R. and R.J.S., manuscript in preparation) and used to immunize sheep. Briefly, chicken breast muscle was extracted in Tris buffer<sup>38</sup> without sucrose and the supernatant fractionated with ammonium sulphate basically as described previously<sup>39</sup>. PGM and LDH were purified from the 40–75% (pH 5.4) and EN from the 75–90% (pH 8.4) ammonium sulphate fraction. PGM was not retained on either phosphocellulose or Cibacron blue columns. It was judged to be 40% pure on SDS-gel electrophoresis, with EN and serum albumin as major contaminants. Its specific activity was 90 U per mg. LDH was retained on phosphocellulose and was eluted with a Tris-HCl gradient and then eluted from a Cibacron blue column with substrate. It was essentially homogeneous by one- and two-dimensional gel electrophoresis, and had a specific activity of 205 U per mg. EN was eluted from phosphocellulose with a Tris-HCl gradient. It was contaminated with TPI and had a specific activity of 66 U per mg. Two 35-mm dish cultures of uninfected chicken embryo fibroblasts<sup>40</sup> were each labelled with 0.5 mCi of <sup>35</sup>S-methionine as described elsewhere<sup>11</sup> and lysed in a total of 1 ml of immunoprecipitation buffer<sup>40</sup>. 90- $\mu$ l samples were immunoprecipitated with 2  $\mu$ l of each antiserum. Immunoprecipitates were collected and washed<sup>40</sup> and one-half of each was dissociated with 15  $\mu$ l of two-dimensional gel sample buffer<sup>41</sup> containing 0.3% SDS. 5  $\mu$ l of each was mixed with 5  $\mu$ l of a sample containing about 25  $\mu$ g total unlabelled chicken fibroblast proteins and separated by isoelectric focusing (mixed pH 6–8 and pH 8–10 ampholytes<sup>21</sup>) and SDS-gel electrophoresis. Gels were stained with silver<sup>42</sup> and impregnated with diphenyloxazole (PPO) (which may not greatly increase the sensitivity<sup>43</sup>), dried and autoradiographed for 7 days. The autoradiographs and stained gels were aligned using radioactive ink marks.

substrate proteins is important for the biological effects of these agents. We and others have sought to identify those cell proteins which become newly phosphorylated at tyrosine after transformation or growth factor treatment, making systematic use of two-dimensional gel electrophoresis<sup>2,5,9–13</sup>. This procedure, coupled with a technique which enriches for phosphate bound to tyrosine<sup>11,13</sup>, has allowed the detection of several such phosphoproteins, but probably many more await discovery<sup>14,15</sup>. The question of which substrates are important for transformation requires a knowledge of their functions and *in vitro* assays for the effects of phosphorylation. The function of the only named substrate to date, vinculin<sup>16</sup>, is not readily assayed *in vitro*. We report here that three proteins phosphorylated at tyrosine in Rous sarcoma virus (RSV)-transformed cells are glycolytic enzymes. It should now be possible to test whether phosphorylation of a substrate at tyrosine affects its function.

### Enolase and phosphoglycerate mutase are phosphorylated at tyrosine

Seven glycolytic enzymes, aldolase (AL, EC 4.1.2.6), triose phosphate isomerase (TPI, EC 5.3.1.1), phosphoglycerate kinase (PGK, EC 2.7.2.3), phosphoglycerate mutase (PGM, EC 2.7.5.3), enolase (EN, EC 4.2.1.11), pyruvate kinase (PK,

EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.27), were purified from chicken breast muscle and used to immunize sheep. An eighth enzyme, glyceraldehyde phosphate dehydrogenase (GAPD, EC 1.2.1.12), had been purified previously<sup>17</sup>. Resultant antisera were capable of immunoprecipitating <sup>35</sup>S-methionine-labelled polypeptides of the appropriate sizes from *in vitro* translation reactions containing chicken breast muscle mRNA (data not shown). However, when used to immunoprecipitate biosynthetically labelled chicken fibroblast proteins, SDS-gel electrophoresis showed that some of the sera precipitated polypeptides in addition to those expected. These may be immunogenic proteins present in small amounts in the purified muscle enzyme preparations used for immunization, but abundant in fibroblast extracts.

Immunoprecipitated <sup>35</sup>S-methionine-labelled chicken fibroblast proteins were analysed by two-dimensional gel electrophoresis. They were compared with proteins of molecular weights (MWs) 28,000 (28K), 39K and 46K which are the nonphosphorylated forms of phosphoproteins that contain phosphotyrosine in RSV-transformed, but not control, chicken fibroblasts<sup>11,18</sup>. Two antisera recognized proteins of interest. One of the proteins that precipitated with sheep anti-EN serum co-migrated on a two-dimensional gel with the 46K protein, which was localized by staining unlabelled chicken fibroblast

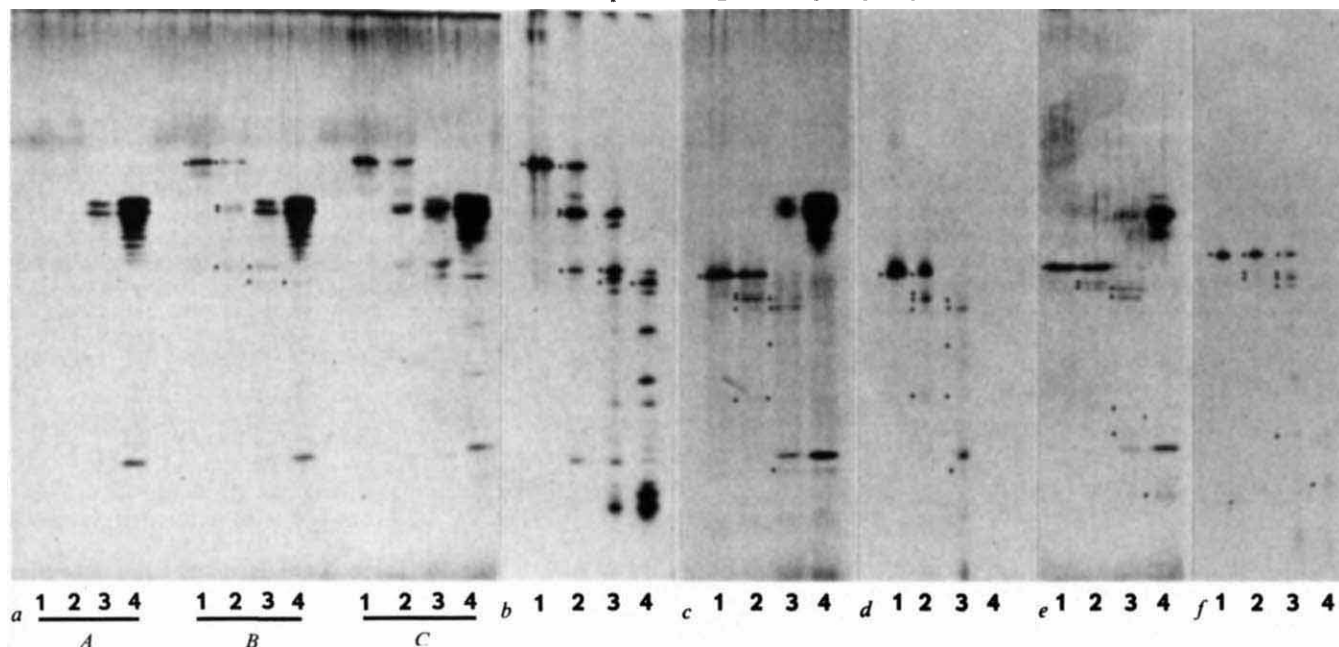
proteins added as internal markers to the gel sample (Fig. 1b, c). Similarly, one of the proteins that precipitated with sheep anti-PGM serum co-migrated with the 28K protein (Fig. 1d, e). In contrast, none of the polypeptides that precipitated with the antisera to the other six enzymes co-migrated with any previously known substrate for tyrosine protein kinases. The two-dimensional gel coordinates of six enzymes are shown in Fig. 1a.

As the antisera were not monospecific, it was important to prove identity of the fibroblast 46K and 28K polypeptides with EN and PGM, respectively. Unphosphorylated polypeptides were prepared from  $^{35}\text{S}$ -methionine-labelled chicken fibroblasts by two-dimensional gel electrophoresis, and compared with purified chicken muscle EN and PGM. The 46K protein and EN had similar, but not identical, mobilities on SDS-polyacrylamide gel electrophoresis (Fig. 2) and the muscle protein was more basic (data not shown). The 28K protein and PGM could not be resolved by one- or two-dimensional gel electrophoresis (Fig. 2 and data not shown). Digestion with various concentrations of protease during electrophoresis<sup>19</sup> generated fragments from EN, located by silver staining, which were similar but not identical to fragments of the 46K protein, as revealed by autoradiography (Fig. 2). The slight differences between muscle and fibroblast EN are probably due to the expressions of different isozymes<sup>20</sup>. Fragments of chicken PGM had equal mobilities to fragments of the 28K protein. A mouse fibroblast 29K tyrosine protein kinase substrate, which is related to the chicken fibroblast 28K protein, was found to be very similar to a mammalian PGM, specifically that from rabbit muscle (Fig. 2).

These data demonstrate close antigenic and structural similarities between muscle EN and PGM and fibroblast pro-

teins previously identified as the nonphosphorylated forms of phosphotyrosine-containing phosphoproteins<sup>18</sup>. These phosphoproteins were detected first by two-dimensional gel electrophoresis of  $^{32}\text{P}$ -labelled chicken fibroblasts, transformed with RSV<sup>11</sup>. Transformation by Esh sarcoma virus, Y73 virus, PRCII virus or Fujinami sarcoma virus was also found to be effective (refs 11, 12 and our unpublished results). Homologous mouse 3T3 cell proteins are phosphorylated at tyrosine following transformation by RSV<sup>12</sup>, Snyder-Theilen feline sarcoma virus (STFeSV) (our unpublished results) or Abelson virus (29K only<sup>12</sup>). Neither protein is phosphorylated when 3T3 cells are treated with growth factors<sup>5</sup>. As final confirmation, we tested whether antisera to EN and PGM could precipitate phosphoproteins.

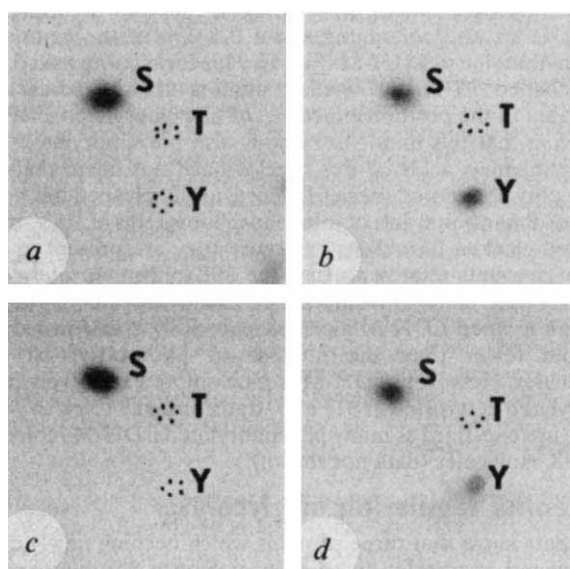
Immunoprecipitates were prepared from normal or RSV-transformed chicken fibroblasts, and phosphoproteins co-migrating with EN and PGM protein markers were extracted from SDS-polyacrylamide gels and partially hydrolysed with acid. Each phosphoenzyme contained phosphoserine in normal cells. We have previously shown that there are forms of the 46K and 28K proteins which contain phosphoserine in normal cells<sup>18</sup>. Phosphotyrosine and phosphoserine were detected in both proteins from RSV-transformed cells (Fig. 3). As a control, similar immunoprecipitations were performed with antisera to other glycolytic enzymes. TPI and PGK seemed not to be phosphorylated. Antibodies to AL, GAPD, PK and LDH precipitated phosphorylated polypeptides of similar apparent sizes to the respective enzymes. AL, GAPD and PK were only weakly phosphorylated in both normal and transformed cells, and contained predominantly phosphoserine and/or phosphothreonine. Surprisingly, LDH from transformed cells contained significant phosphotyrosine (see below).



**Fig. 2** Comparative one-dimensional peptide maps of glycolytic enzymes from muscle and of fibroblast proteins isolated from two-dimensional gels. *a*, Stained SDS-polyacrylamide gel. Four different amounts of *Staphylococcus aureus* V8 protease (0, 5, 50 and 500 ng; 1 to 4 respectively) were loaded into three gel tracks each. Each track in sets *B* and *C* also contained about 100 ng of purified chicken muscle EN. In addition, each track in *C* contained a piece of a two-dimensional gel containing  $^{35}\text{S}$ -methionine-labelled chicken fibroblast 46K protein. Stained proteolytic cleavage products of EN were identified by comparing tracks in *A* and *B*, and are marked by dots. *b*, Autoradiograph of tracks *C* from the gel shown in *a*. *c*, Stained SDS-polyacrylamide gel. Tracks *B* from an experiment performed exactly as described above but with about 250 ng of purified chicken muscle PGM instead of EN, and with two-dimensional gel pieces containing chicken fibroblast 28K protein instead of 46K protein. *d*, Autoradiograph of tracks *C* from the experiment described in *c*. *e*, Stained SDS-polyacrylamide gel. Tracks *B* from an experiment performed exactly as described above but with about 250 ng of purified rabbit muscle PGM (Boehringer-Mannheim 108464) instead of EN, and with two-dimensional gel pieces containing mouse 3T3 cell 28K protein instead of 46K protein. *f*, Autoradiograph of tracks set *C* from the experiment described in *e*.

**Methods:** Nonradioactive purified enzymes were obtained free of ammonium sulphate by acid precipitation with tRNA carrier. They were dissolved by boiling in SDS gel sample buffer containing 2% SDS<sup>12</sup>, lyophilized to remove mercaptoethanol, and dissolved in 20 volumes of gel-swelling buffer<sup>19</sup>. Analysis was according to Cleveland *et al.*<sup>19</sup>, except that buffers contained 1 mM mercaptoethanol and the gel was 20% acrylamide and 0.065% bisacrylamide. Gels were stained<sup>42</sup>, impregnated with PPO and exposed for 7 days. They were aligned using radioactive ink marks. Some small fragments which could be detected only by autoradiography may be particularly methionine-rich, could bind silver poorly, or could reflect genuine structural differences.





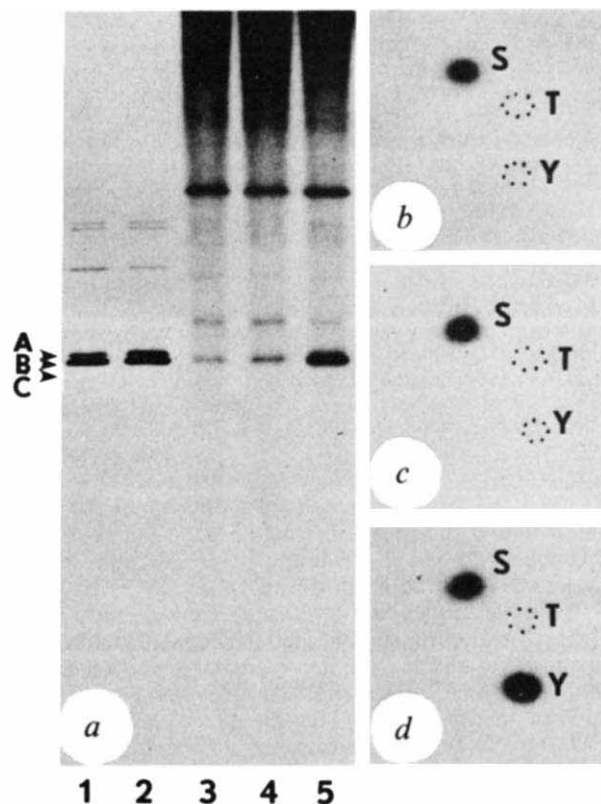
**Fig. 3** Phosphoamino acid compositions of 46K and 28K proteins precipitated with anti-EN and anti-PGM sera. *a*, 46K protein from control cells. *b*, 46K protein from RSV-transformed cells. *c*, 28K protein from control cells. *d*, 28K protein from RSV-transformed cells.

**Methods:** Control or RSV-transformed chicken fibroblast cultures were labelled with 2.5 mCi (*a*, *b*) or 1 mCi (*c*, *d*) of  $H_3^{32}PO_4$  in 1 ml of the medium described<sup>11</sup> for 18 h. For anti-PGM serum, cultures were lysed in the presence of 2 mM EDTA and immunoprecipitated as for Fig. 1. For anti-EN serum, nucleic acid was first removed from the samples<sup>44</sup> by lysis in pH 7.8 buffer<sup>12</sup> supplemented with 20 mM  $Na_4P_2O_7$  (ref. 48) and 25 mg of DE52 cellulose (Whatman). After vigorous mixing the cellulose was removed with a 1-min spin in a microcentrifuge and the supernatant was adjusted to the usual NaCl and detergent concentrations for clarification and immunoprecipitation<sup>40</sup>. Immunoprecipitates were dissociated in SDS-gel sample buffer<sup>12</sup> and analysed on SDS-polyacrylamide gels.  $^{32}P$ -labelled bands co-migrating with  $^{35}S$ -methionine EN or PGM markers were excised, eluted and the phosphoproteins hydrolysed in 5.7 M HCl for 1 h at 110°C<sup>45,46</sup>. Phosphoamino acids were separated by two-dimensional electrophoresis in cellulose thin layers<sup>47</sup> and exposed to film with a fluorescent screen for 3 days. S, T, Y, respective positions of nonradioactive phosphoserine, phosphothreonine and phosphotyrosine mixed with each sample.

The 46K and 28K proteins are both abundant<sup>18</sup>, soluble, cytoplasmic<sup>21</sup> proteins, consistent with their identification as glycolytic enzymes. Polypeptides antigenically related to the 46K protein have been found in every tissue studied (K. Gould and J.A.C., unpublished data). EN has not previously been shown to be a phosphoprotein, but the catalytic form of PGM is known to be a phosphoprotein<sup>22,23</sup>. On incubation *in vitro* with 2,3-diphosphoglyceric acid (2,3-diPGA), PGM becomes phosphorylated in an acid-labile linkage to histidine<sup>24</sup>. Because it was possible that the phosphoserine and/or phosphotyrosine we detected in PGM *in vivo* arose by phosphate transfer from histidine during acid hydrolysis<sup>25</sup>, we incubated rabbit muscle PGM with  $^{32}P$ -2,3-diPGA<sup>24</sup>. Phosphodistidine was released after alkaline hydrolysis of the  $^{32}P$ -PGM. However, on acid hydrolysis in our standard conditions, no phosphoamino acids were detected (data not shown). Thus, it is likely that the detection of phosphotyrosine and phosphoserine in PGM is due to the actions of specific protein kinases rather than an artefact of hydrolysis.

### Lactate dehydrogenase is phosphorylated at tyrosine

Phosphorylated LDH was detected by immunoprecipitation, even though no 35 K phosphoprotein which could be phos-



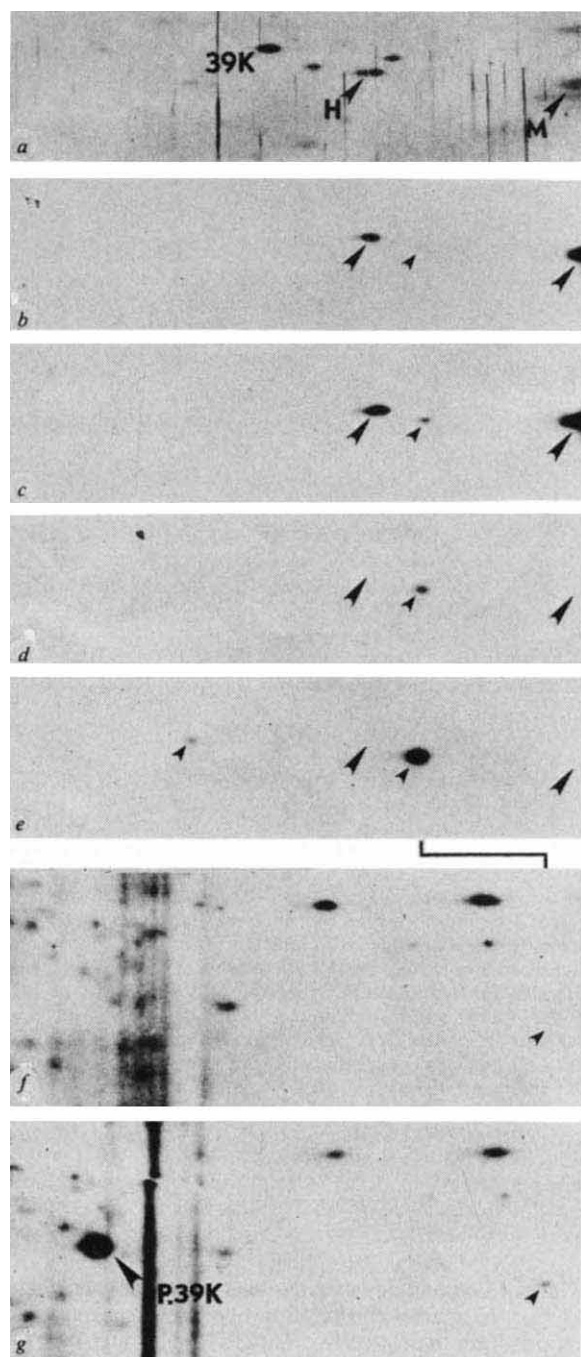
**Fig. 4** Phosphorylation of LDH. *a*, SDS-polyacrylamide gel of immunoprecipitates. Tracks 1, 2,  $^{35}S$ -methionine; tracks 3–5,  $^{32}P$ ; tracks 1, 3, uninfected; track 4, *td* 108; tracks 2, 5, RSV-transformed. *b–d*, Phosphoamino acid analyses. Phosphorylated LDH-M from uninfected (*b*), *td* 108 infected (*c*) or RSV-transformed (*d*) chicken fibroblasts.

**Methods:** Chicken fibroblasts were infected with wild-type RSV (SR-RSV-A) or a transformation defective mutant (*td* 108). Cultures were labelled with 0.5 mCi  $^{35}S$ -methionine or 2 mCi  $^{32}P$  for 15 h. Cell lysates were treated with DE52 cellulose (Fig. 3) and immunoprecipitated with anti-LDH serum. In *a*, Immunoprecipitates from 1/20th of each culture were analysed on a 17.5% acrylamide 0.073% bisacrylamide gel. The following standards were run on the same gel; A, chicken heart muscle LDH; B, chicken breast muscle LDH (Sigma L-9005); C, pigeon breast muscle MDH (Sigma M-7508). Phosphorylated LDH-M samples from a similar polyacrylamide gel were hydrolysed in acid and analysed as described above (Fig. 3).

phorylated LDH had been found by two-dimensional gel electrophoresis. In normal chicken fibroblasts, and in cells infected with transformation-defective mutant RSV, LDH was phosphorylated at low level, mostly at serine (Fig. 4). The LDH of fibroblasts infected with RSV was more extensively phosphorylated (Fig. 4), and the phosphate was retained through alkali treatment of the gel (data not shown). The immunoprecipitated protein contained phosphotyrosine and phosphoserine (Fig. 4).

To characterize which forms of LDH were phosphorylated, immunoprecipitated extracts of  $^{35}S$ -methionine-labelled normal or RSV-transformed chicken fibroblasts were analysed on SDS-polyacrylamide gels (Fig. 4). Two polypeptides were detected which were not found with any of the other sera. The major polypeptide co-migrated with the chicken muscle-specific isozyme subunit of LDH (LDH-M). The minor, slightly slower-migrating polypeptide co-migrated with the chicken heart isozyme, LDH-H. Moreover, one-dimensional peptide mapping<sup>19</sup> showed that the major immunoprecipitated fibroblast protein was indistinguishable from our preparation of chicken muscle LDH-M, but was quite different from chicken heart LDH-H (data not shown).

Two-dimensional gel electrophoresis of immunoprecipitated LDH showed LDH-M to be more basic than LDH-H, as even though no 35K phosphoprotein which could be phos-



**Fig. 5** Two-dimensional gel electrophoresis of LDH. *a-e*, Two-dimensional gels of immunoprecipitated LDH. *b*,  $^{35}\text{S}$ -methionine; *d*,  $^{32}\text{P}$ -labelled control cells; *c*,  $^{35}\text{S}$ -methionine; *e*,  $^{32}\text{P}$ -labelled RSV-transformed cells. One-fifteenth of each of the immunoprecipitates described (Fig. 4) was mixed with 25  $\mu\text{g}$  non-radioactive total chicken fibroblast proteins and analysed (Fig. 1). A region from the basic end of each gel is shown. *a*, Typical staining pattern; *b-e*, autoradiographs (15 h exposures with intensifying screens). *f*, *g*, Two-dimensional gels of total cellular phosphoproteins. Uninfected (*f*) or RSV-transformed (*g*) cultures were labelled with 0.5 mCi  $^{32}\text{P}$  and 1/10th of each was prepared directly for two-dimensional gel electrophoresis<sup>11</sup>. Gels were incubated in alkali<sup>11</sup> and exposed with an intensifying screen for 16 h. Phosphorylated LDH-M could also be detected among total RSV-transformed cell phosphoproteins by autoradiography before alkali treatment of the gel, or by analysis of total proteins, labelled with  $^{35}\text{S}$ -methionine (data not shown). Large arrowheads indicate positions of LDH-H (left) and LDH-M (right) and small arrowheads their phosphorylated forms. P.39K, the 39K phosphoprotein.

chicken fibroblasts, a relatively increased amount of a third polypeptide was detected, more acidic than but the same size as LDH-M, and containing about 0.5–1% of the amount of  $^{35}\text{S}$ -methionine in LDH-M (Fig. 5). This form co-migrated with  $^{32}\text{P}$ -labelled LDH. A smaller amount of  $^{32}\text{P}$  radioactivity migrated in the position expected for phosphorylated LDH-H, implying that this minor isozyme is also phosphorylated. The phosphoamino acids of this species have not been analysed. Both phosphorylated species had previously escaped our notice in two-dimensional gels of total phosphoproteins of RSV-transformed chicken fibroblasts<sup>11</sup> because they are present in very minor amounts relative to, say, the 39K phosphoprotein (Fig. 5f). Indeed, there are only of the order of 50,000–100,000 phosphorylated LDH-M molecules per RSV-transformed cell, 50-fold fewer than the number of phosphorylated 39K molecules. However, LDH-M is much more extensively phosphorylated at tyrosine in STFeSV-transformed 3T3 cells, where there are one-third as many phosphorylated LDH-M molecules as 39K molecules (data not shown).

### Potential regulation of glycolysis

Our data show that three proteins which become newly phosphorylated at tyrosine in chicken or mouse fibroblasts transformed by RSV are immunologically and structurally closely related to, and in all likelihood have the same functions as, the muscle glycolytic enzymes EN, PGM and LDH. For PGM and LDH, the fibroblast proteins cannot be distinguished from the muscle isozymes. In the case of EN, the slight differences in apparent molecular size and structure can be attributed to the existence of different isozymes in fibroblasts and muscle cells. For the first time, we are now in a position to set up *in vitro* systems to study the effects of tyrosine phosphorylation on the function of a substrate protein.

What could be the role of phosphorylation of EN, PGM and LDH at tyrosine? These enzymes catalyse three out of the last four steps of glycolysis. The enzymes catalysing the other five glycolytic steps from fructose-1,6-diphosphate to lactic acid do not seem to be so extensively phosphorylated on tyrosine. Without appropriate antisera, we have been unable to examine the first three enzymes of glycolysis for phosphorylation, although in the light of our results it becomes important to do so. We have also been unable to confirm a report<sup>27</sup> that another major tyrosine kinase substrate (39K protein, Fig. 1) is identical with cytosolic malate dehydrogenase (MDH). We do not believe that we have mis-identified MDH as LDH, as we can resolve MDH from LDH on our gels (Fig. 4a) and it has a distinctive peptide map (data not shown). Moreover, because MDH seems smaller than LDH-M, it is clearly even smaller than the 39K protein (Figs 1a, 5f).

Tyrosine phosphorylation of EN, PGM and LDH could be gratuitous, or it could contribute to the high rate of aerobic glycolysis, evident from the rapid glucose uptake and lactate production of RSV-transformed cells<sup>28–30</sup>. In a survey of RSV mutants, we have found that phosphorylation of EN and PGM correlates more significantly with increased hexose transport rate (that is, increased glycolysis) than with other parameters of transformation<sup>31</sup>. However, only about 5% of the EN and PGM isozymes become phosphorylated on transformation with wild-type RSV<sup>18</sup>. Another substrate, vinculin, is phosphorylated to about 1%<sup>16</sup>. EN and PGM are dimers and LDH is a tetramer<sup>24,32,33</sup>, so at most ~10% of enzyme molecules could be modified at steady state. Potentially, however, a combination of kinase and phosphatase action could cycle every molecule through the phosphorylated state. Existing evidence points to hexose transport, phosphofructokinase and PK, rather than EN or PGM, as the key regulatory steps for glycolysis in RSV-transformed cells<sup>34,35,36</sup>. Indeed, the levels of substrates for EN and PGM are not reduced, suggesting that these enzymes are not activated<sup>29,30</sup>.

It is possible, however, that phosphorylation of these enzymes could contribute less obviously to increased glycolysis. Besides possible effects on substrate binding or catalysis, or on binding



of allosteric effectors, phosphorylation could modify the organization of glycolytic enzymes inside the cell (reviewed in ref. 37). Potentially, phosphorylation of only a small fraction of molecules could have large effects on their spatial organization, and thereby on glycolysis.

The interactions between enzyme tyrosine phosphorylation and glycolysis may be complex. If evidence of regulation is found, it may help to explain the high rates of glycolysis in fibroblasts transformed by retroviruses containing *src*, *yes*, *abl*, *fps* or *fes* genes, for which we have evidence that the EN and

PGM isozymes are phosphorylated<sup>12</sup>. Although we do not yet know the phosphorylation state of LDH in many conditions, it is clear that EN and PGM are not phosphorylated on tyrosine in fibroblasts transformed by many agents<sup>12</sup>, implying that there must be other ways in which glycolysis in tumours is regulated.

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# A transcriptional function for the repetitive ribosomal spacer in *Xenopus laevis*

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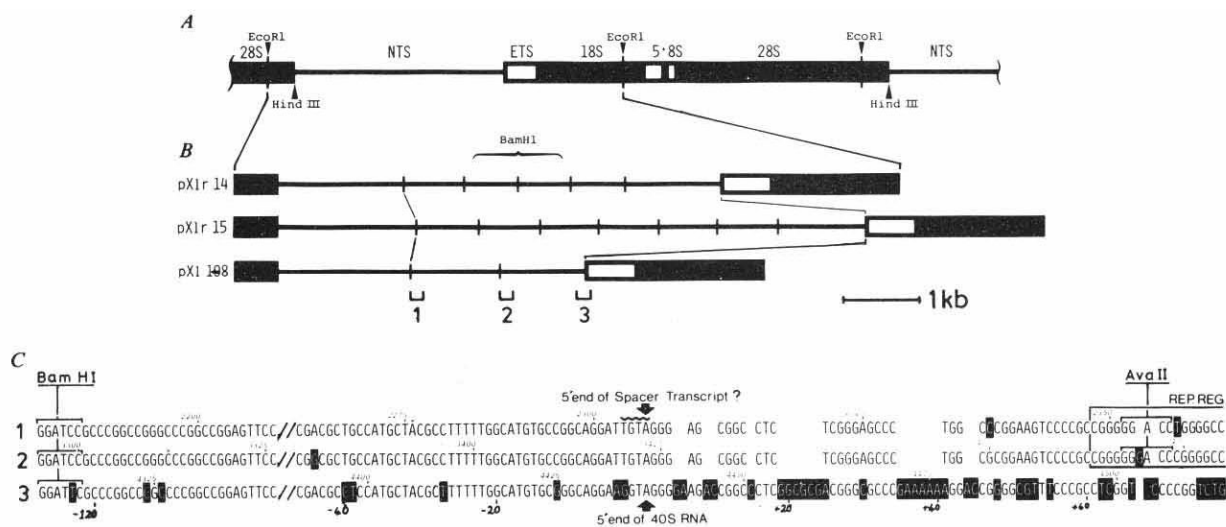
*The function of the Xenopus laevis ribosomal spacer has been studied in vivo and by microinjection of in vitro mutants into Xenopus oocytes. It is shown that the spacer directs specific RNA transcripts which most probably terminate upstream of the ribosomal genes and that it is able to modulate transcription of these genes. The data lead to a model in which the ribosomal spacer is a loading site for RNA polymerase I and spacer transcription is the driving force by which polymerase is delivered to the ribosomal gene promoter.*

A COMMON feature of the organization of most eukaryotic ribosomal operons is their tandem repetition at one or more sites in the genome. As a result of this repetition, each operon is separated from its neighbours by a segment of noncoding DNA referred to as the ribosomal spacer<sup>1</sup>. In *Xenopus laevis* and many other eukaryotes the bulk of the ribosomal spacer is not transcribed as part of the major species of ribosomal precursor RNA (pre-rRNA) and is therefore referred to as the non-transcribed spacer (NTS) (Fig. 1A). In both *X. laevis* and *Xenopus borealis*, the NTS is heterogeneous in length (for examples see Fig. 1B) and is mainly composed of short repeated DNA sequences<sup>2–4</sup>. This sequence composition stimulated speculation that the NTS may in some way be involved in the parallel evolution of a given family of ribosomal genes<sup>2</sup> or simply be a self propagating relic of evolution. However, more detailed studies of a *X. laevis* ribosomal spacer<sup>5–7</sup> and recently of *X. borealis*, *Xenopus clivii* and *Drosophila melanogaster* ribosomal spacers<sup>8,9</sup>, have suggested an active transcriptional role

for the NTS. These studies have shown that a common feature of all NTS regions is the reduplication of the pre-rRNA initiation site sequence (see Fig. 1C). By mapping a *X. laevis* ribosomal promoter<sup>10</sup> it was also possible to demonstrate that the 40S pre-rRNA initiation site reduplications found in the *X. laevis* NTS are indeed promoter reduplications. The present work identifies the transcripts of the *X. laevis* ribosomal 'NTS' directed by these reduplicated promoters, determines the manner of the transcription and presents evidence demonstrating that the NTS is a functional element in 40S pre-rRNA transcription.

## Active promoters in the *X. laevis* ribosomal spacer

A generalized ribosomal gene unit from *X. laevis*, giving the arrangement of coding and spacer sequences, is shown in Fig. 1A. The most extensively studied example of a *X. laevis* ribosomal spacer is that of the cloned *EcoRI* fragment contained



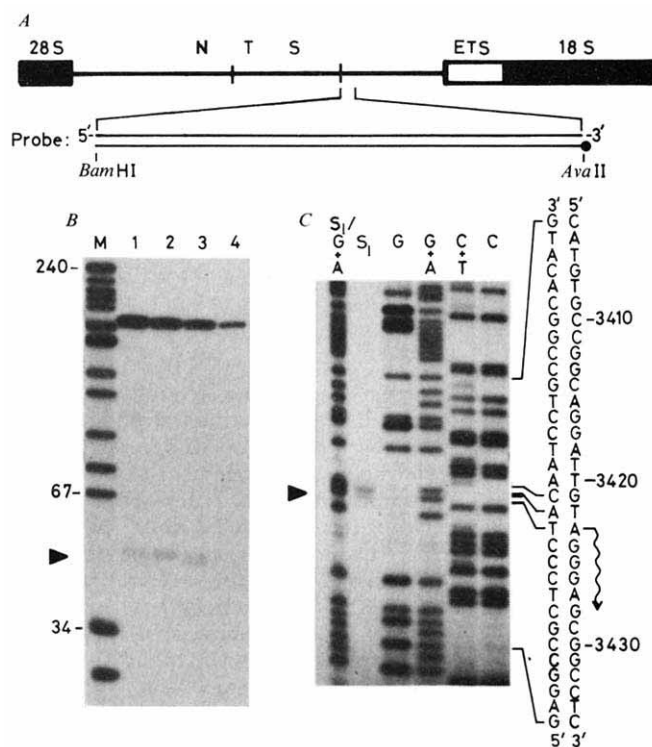
**Fig. 1** The organization of the ribosomal genes in *X. laevis*. **A**, The gene repeat; **B**, spacer fragments from three independently cloned ribosomal genes<sup>4,5</sup>; **C**, base sequences of: 1, *Bam* island I; 2, *Bam* island II; 3, the 40S promoter of pXI108. The positions of these sequences on pXI108 are indicated in **B**. Sequence homology has been optimized where possible by the introduction of spaces and nonhomologous bases are shown as white on black. In **A** and **B** boxed regions indicate transcribed sequences and solid boxes, gene sequences; NTS, non-transcribed spacer; ETS, external transcribed spacer; REP, REP., repetitive regions 2 and 3. *Eco*RI, *Hind*III, *Bam*HI and *Ava*II indicate restriction enzyme cutting sites. The sequence numbering in **C** is, above the line, taken from ref. 7 and, below the line, with reference to the 40S initiation site as +1.

in the plasmids pXI108 (ref. 5) and pXI108c (ref. 10) (Fig. 1B). The spacer region of this isolate has been almost entirely base sequenced<sup>5-7</sup> and the presence of 40S pre-rRNA initiation site duplications identified approximately 1 and 2 kilobase pairs (kbp) upstream of the mapped site of 40S pre-rRNA initiation. The duplicated sequences, referred to as '*Bam* islands'<sup>5</sup>, could be identified by their single *Bam*HI restriction site (Fig. 1B, C).

The observed transcription of the *X. laevis* ribosomal NTS<sup>6,11-16</sup> is consistent with *Bam* island promoter activity, but the techniques used do not allow further conclusions to be drawn. I therefore began the present study by determining the

origin of the NTS transcripts produced in a *X. laevis* tissue culture line<sup>6</sup>. The approach used was to analyse total RNA from these cells by the S<sub>1</sub>-mapping technique<sup>17</sup>. A hybridization probe was obtained from the *Bam* island II of pXI108 (Fig. 2A) and purified by cloning. Comparison of the sequence of this *Bam*HI to *Ava*II probe with the sequence of the 40S initiation site (Fig. 1C) shows that within the 40S transcribed region the two sequences are extremely divergent and thus that the probe chosen was specific for NTS transcripts. When the total tissue culture RNA was mapped with this probe, 5'-end-labelled at the *Ava*II site, a single S<sub>1</sub>-protected fragment was

**Fig. 2** The 5' terminus of the NTS transcripts. **A**, The hybridization probe. The *Bam*HI-*Ava*II fragment from *Bam* island II of pXI108 was cloned between the *Bam*HI and an *Ava*II site in the tetracycline gene of pBR322<sup>22</sup>, to produce the plasmid pXINS1. The fragment was recovered by restricting pXINS1 with *Ava*II, dephosphorylating the *Ava*II ends with alkaline phosphatase and then restricting with *Bam*HI. It was then purified by gel electrophoresis on low-melting point agarose (Sigma) and 5'-end-labelled at the *Ava*II site only, using T4 polynucleotide kinase and excess [ $\gamma$ -<sup>32</sup>P]ATP (>5,000 Ci mmol<sup>-1</sup>). The enzymes were obtained from Boehringer-Mannheim, New England Biolabs, Bethesda Research Laboratories and PL Biochemicals and used as recommended by the supplier. In the diagram the solid circle indicates the position of the <sup>32</sup>P label. **B**, Approximately 0.05 pmol of the 5'-end-labelled probe ( $\geq 10,000$  c.p.m.) was hybridized for 12 h at 42 °C with 100  $\mu$ g of total cellular RNA in 50  $\mu$ l of 80% formamide, 0.4 M NaCl, 40 mM MOPS, pH 6.7. The reactions were stopped by adding 450  $\mu$ l S<sub>1</sub> buffer containing S<sub>1</sub> nuclease and samples incubated at 37 °C for 45 min. Protected fragments were analysed on an 8% polyacrylamide gel in 8 M urea, 90 mM Tris-borate, 2 mM EDTA and detected by autoradiography at -70 °C using an intensifying screen (Ilford, DuPont). It was estimated from the known input c.p.m. of molecular weight marker (~30 c.p.m. per band) that 3% or less of the DNA probe was protected by RNA in each S<sub>1</sub> reaction. Thus, a >30 $\times$  DNA excess was achieved in all S<sub>1</sub> mapping experiments here and in subsequent figures. Lanes 1, 2 and 3 show the S<sub>1</sub> mapping of *X. laevis* tissue culture RNA<sup>6</sup> using, respectively, 1, 5 and 25 units of S<sub>1</sub> nuclease and lane 4 the S<sub>1</sub> mapping of *Escherichia coli* RNA using 5 units of S<sub>1</sub> nuclease. Lane M, *Hpa*II fragments of pBR322 with sizes in bp. **C**, The protected fragment shown in lane 2 of **B** was electrophoresed in parallel with the sequence ladder of the probe and was either mixed with one base-specific reaction (S<sub>1</sub>/G + A) or analysed alone (S<sub>1</sub>). G, G + A, C + T and C refer to the base specificities of the reactions used<sup>23</sup>. A correction of slightly more than one base has been made in correlating the length of the protected fragments with the sequence ladder<sup>6</sup>. Sequence numbering<sup>7</sup> is that given in Fig. 1. Solid arrowheads in **B** and **C** indicate the positions of S<sub>1</sub>-protected fragments.





detected (Fig. 2B). This fragment was of the size expected if the NTS transcripts had been initiated *in vivo* at *Bam* island promoters. Analysis of the  $S_1$ -protected fragment in parallel with the sequence ladder of the probe (Fig. 2C) demonstrated that the 5' termini of the NTS transcripts map exactly to the sites predicted for *Bam* island initiation (+3, -0 bp) (see also Fig. 1C). Thus, it seems clear that *in vivo*, the *Bam* island sequences actively promote ribosomal spacer transcription. In support of this conclusion, deletion of the *Bam* islands essentially stops NTS transcription (see below).

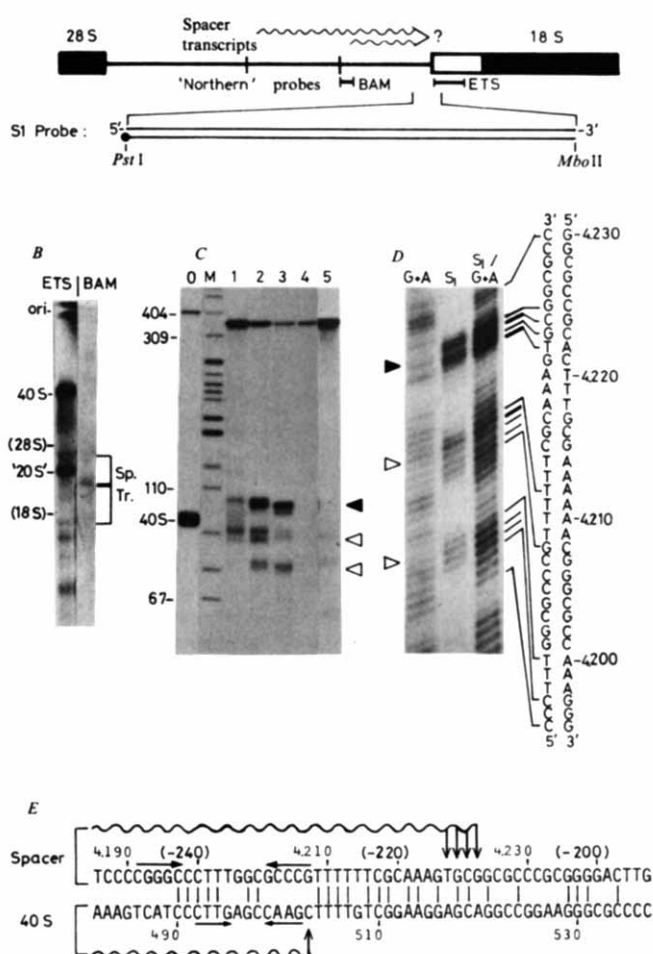
### The 3' terminus of the spacer transcripts

NTS transcripts initiated at the *Bam* island promoters could have several possible fates. They could read through into the 40S pre-rRNA coding region to produce a long precursor RNA which may or may not then be processed to the mature 40S pre-rRNA. NTS transcripts might, however, terminate upstream of the 40S pre-rRNA initiation site. To distinguish the processing and termination possibilities from simple read-through, two experiments were performed. First the cloned *Bam* island fragment (Fig. 2A) and a cloned fragment from the external transcribed spacer (ETS) of pX1108 (Fig. 3A) were independently used to probe electrophoretically fractionated total tissue culture RNA transferred to nitrocellulose (Northern

blot, Fig. 3B). The 40S pre-rRNA and its processing intermediates were clearly detected with the ETS probe and at least one RNA species 2-3 kb in length was weakly detected with the *Bam* island probe. However, no transcripts longer than 40S pre-rRNA were detected with either probe (Fig. 3B). This result suggested that NTS transcripts were terminated or processed within the ribosomal spacer.

If the NTS transcripts were terminated or processed, the sequence organization of the ribosomal spacer<sup>7</sup> suggested that this might occur near the NTS-ETS boundary. The size of the NTS transcripts identified in Fig. 3B was also consistent with this possibility, as the average spacing of *Bam* island promoters and the NTS-ETS boundary must be 2-3 kbp (Fig. 1B). When a 3'-end-labelled probe from the NTS-ETS boundary of pX1108 (Fig. 3A) was used to probe total RNA for 3' termini of the NTS transcripts, three closely spaced bands of  $S_1$  protection were detected (Fig. 3C). It was deduced (see Fig. 3 legend) that the lower two of these bands were probably artefactual, being due to  $S_1$  digestion within unstable dA-rU hybrids proximal to a true RNA terminus. The upper band of probe protection (Fig. 3C), however, maps a true 3' terminus of the NTS transcripts. Electrophoretic comparison of this band of probe protection with the sequence ladder of the probe (Fig. 3D) identified the site at which the NTS transcripts terminate

**Fig. 3** The 3' terminus of the NTS transcripts. **A**, The hybridization probes from pX1108 are indicated by the solid bars ('Northern' probes) or by expansion of the relevant region ( $S_1$  probe). The Northern probes were both purified by cloning, 'BAM' being contained in the plasmid pXINS1 described in Fig. 2 legend. 'ETS' was an *Mbo*II to *Bgl*I fragment from the external transcribed spacer (ETS) of pX1108<sup>7</sup> and was inserted into the *Eco*RI site of pBR322 by flush end ligation<sup>10</sup> to produce the plasmid pXIES1. To obtain the  $S_1$  probe, 1 pmol of a *Pst*I-(*Mbo*II)-*Taq*I fragment was 3'-end-labelled using 8 units of terminal transferase (Tdt, BRL) and 50  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]GTP (3,000 Ci mmol<sup>-1</sup>, Amersham) in 10  $\mu$ l, 100 mM Na cacodylate pH 7, 1 mM CoCl<sub>2</sub> for 1.5 h at 37 °C and subsequently treated with 400 units RNase T<sub>1</sub> (Sigma) for 1 h at 37 °C. The fragment was then restricted with *Mbo*II and the *Pst*I-*Mbo*II fragment purified by gel electrophoresis. The solid circle indicates the position of the <sup>32</sup>P label. **B**, 20  $\mu$ g of total *X. laevis* tissue culture RNA was fractionated on a formaldehyde-agarose gel and transferred to 0.1  $\mu$ m nitrocellulose (Schleicher & Schüll) (G. Schutz, personal communication) in duplicate. Each transfer was then hybridized<sup>24</sup> with either the plasmid pXINS1 (BAM) or the plasmid pXIES1 (ETS), which had been <sup>32</sup>P-labelled by nick-translation<sup>25</sup> ( $\sim 5 \times 10^8$  d.p.m. per  $\mu$ g at  $\sim 1 \times 10^6$  d.p.m. ml<sup>-1</sup>) and autoradiographed as in Fig. 2. The 40S and '20S' pre-rRNAs are, respectively, 8.0 and 3.2  $\pm$  0.2 kb (T. M., unpublished results) and the 18S rRNA is 1.825 kb<sup>26</sup> in length. 'Sp.Tr.' indicates the positions of spacer transcription products. **C**, Total cellular RNA was analysed by  $S_1$  mapping as described in Fig. 2 legend, except for lane 0 where the hybridization and  $S_1$  digestion were performed at 57 and 45 °C, respectively<sup>6</sup>. Lanes 1, 2 and 3, *X. laevis* tissue culture RNA, using respectively 1, 5 and 25 units  $S_1$  nuclease and lane 4 *E. coli* RNA, using 5 units  $S_1$  nuclease, were mapped with the 3'-end-labelled probe shown in A. Lane 5 is an extensive overexposure of lane 4. Lane 0, 20  $\mu$ g of *X. laevis* tissue culture RNA, mapped with the 5'-end-labelled probe used in Fig. 4C and previously to detect the 40S pre-rRNA 5' terminus<sup>10</sup>. Lane M, *Hpa*II fragments of pBR322 with sizes in bp. **D**, Lane G+A, ladder of G and A-specific cleavage<sup>23</sup> of the 3'-labelled probe shown in A. Lane  $S_1$ , the  $S_1$ -protected fragments of lane 2 in C. Lane  $S_1$ /G+A, mixture of fragments from lanes G+A and  $S_1$ . A correction of approximately one base was made in correlating the protected fragments with the G+A ladder<sup>6</sup>. Solid and open arrowheads in C and D indicate the positions of the  $S_1$  protected fragments. It was concluded (see also text) that the two shorter transcripts mapped in C and D (open arrowheads) were most probably artefactual, being due to  $S_1$  attack within the longest transcript (solid arrowhead). This conclusion was based on the following observations. Lane 1 in C demonstrates that with slight  $S_1$  digestion essentially two bands of probe protection were seen. Very extensive  $S_1$  digestion (lane 3 in C), however, enhanced only the upper of these two bands and at intermediate digestion (lane 2 in C) this was also the predominant band. As the lower two bands (open arrowheads in C and D) correspond to RNA termini lying within very unstable rU-dA hybrids<sup>27</sup>, it was very likely that they had been produced by  $S_1$  attack within the longer hybrid (solid arrowhead). This was further supported by the observation that in the complete absence of homologous RNA (lane 5 in C), two weak bands of probe protection (open arrowheads) were seen, which must have arisen by  $S_1$  attack within the dA-dT tracts. **E**, The DNA base sequence at the 3' terminus of the NTS transcripts, (spacer) and of the 40S pre-rRNA<sup>7,18</sup> on pX1108. Vertical arrows indicate the mapped termini and horizontal arrows palindromic sequences. Sequence numbering in D and E is that used in Figs 1 and 2 and previously<sup>7</sup>. In both A and E, wavy lines indicate transcripts.



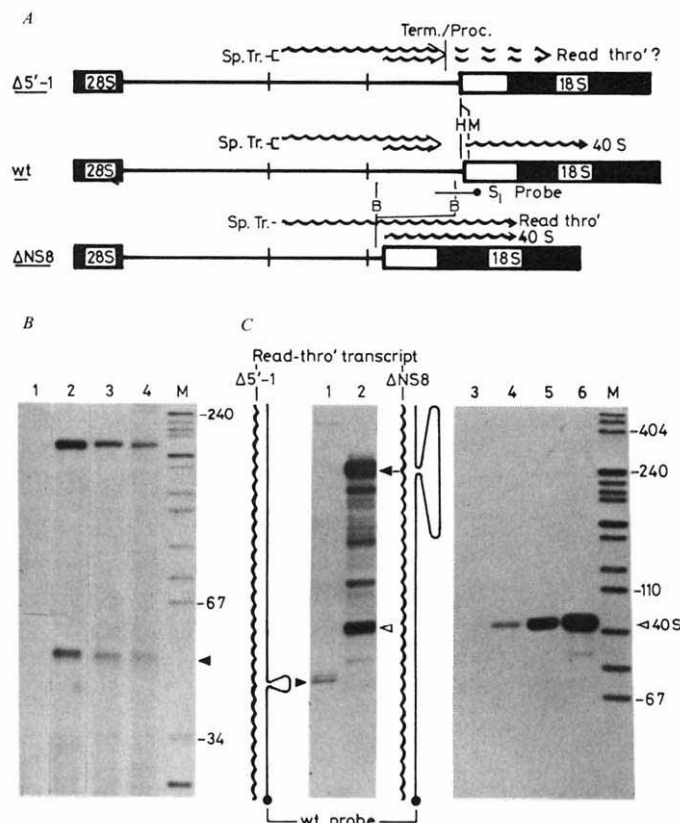
or are processed. This site lies  $213 \pm 2$  bp upstream of the 40S pre-rRNA initiation site (Fig. 3E) and thus outside the 40S promoter<sup>10</sup>.

## Spacer transcripts terminate upstream of the 40S promoter

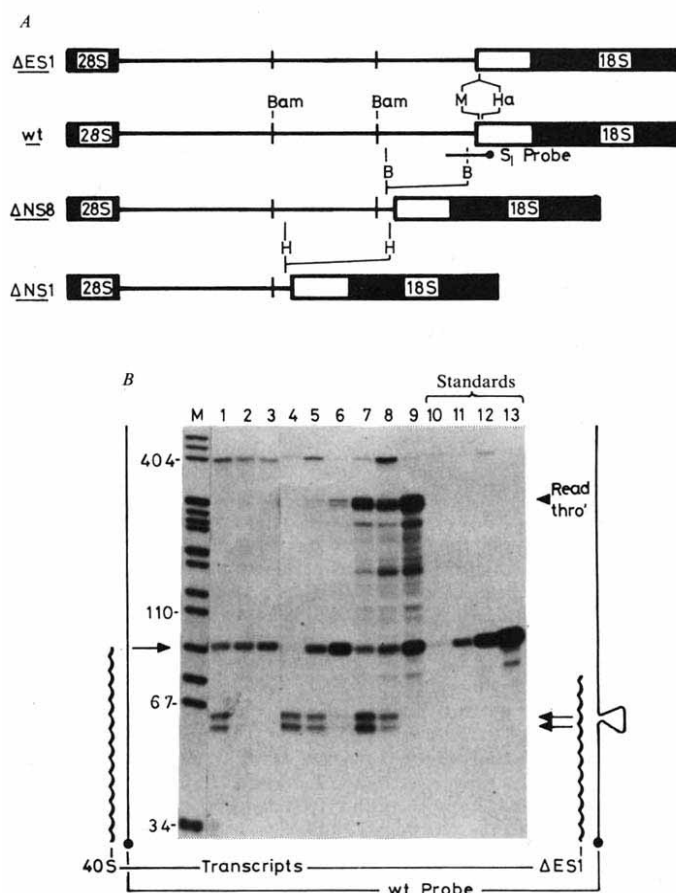
To decide between termination and processing of NTS transcripts at the -213 bp site, it was necessary to determine whether or not sequences downstream of this site were transcribed. Consistent with the termination of NTS transcripts, no significant protection of the probe used to map the 40S pre-rRNA 5' terminus was detected upstream at the -213 bp site (305 bp in Fig. 3C). However, a processed spacer transcript could be rapidly degraded and for this reason would not be

detected. To investigate this problem more thoroughly, I therefore studied the transcription of *in vitro* manipulated ribosomal DNA microinjected into *Xenopus* oocytes.

Several deletion mutations of the pX1108c ribosomal plasmid have recently been analysed by microinjection into *X. borealis* oocytes<sup>10</sup>. It was shown that deletions mapping at or near the 40S initiation site totally abolished 40S promotion. Analysis of the NTS transcription from one such mutant, pX1Δ5'-1, in which the 40S initiation site was deleted (Fig. 4A) demonstrated that the *Bam* islands still actively promoted NTS transcription (Fig. 4B). Thus, using this mutant, NTS transcripts reading through the -213 bp site into the pseudo-stable 40S transcribed region could be detected without interference from the normal 40S transcript. For comparison, the readthrough transcription occurring on pX1ΔNS8 (Fig. 4A), in which the -213 bp site



**Fig. 4** Analysis of spacer transcription from ribosomal mutants microinjected into *X. borealis* oocytes. **A**, The structure of the ribosomal DNA fragment in pX1108c (wt) and spacer deletion mutants pX1Δ5'-1 (Δ5'-1) and pX1ΔNS8 (ΔNS8). Both the construction of these plasmids and their base sequence have been described previously<sup>10</sup>. Transcripts or putative transcripts are shown as wavy or broken wavy lines respectively; Sp.Tr., spacer transcript; 40S, the 40S pre-rRNA. The positions of restriction sites used in construction of the mutants are indicated by: H, *Hga*I; M, *Mbo*II; B, *Bgl*I. 'S<sub>1</sub> probe' refers to the 5'-end-labelled probe used in **C** below, the solid circle indicates the position of the <sup>32</sup>P label. **B**, Total cellular RNA was S<sub>1</sub> mapped as in Fig. 2 using the same 5'-end-labelled probe shown there, but 5 units of S<sub>1</sub> nuclease and 25 μg of RNA were used. Lane 1, *X. borealis* oocyte RNA; lane 2, *X. laevis* tissue culture RNA; lanes 3, 4, RNA from *X. borealis* oocytes microinjected with, respectively, 250 pg pX1Δ5'-1 or the molar equivalent of pX1ΔNS8, both in the presence of ~10 μg ml<sup>-1</sup> α-amanitin (BCL) (final oocyte concentration). **C**, Total oocyte RNA (25 μg) was S<sub>1</sub> mapped as in Fig. 2, but the probe shown in **A** above was used, hybridization was carried out at 59 °C and S<sub>1</sub> digestion was carried out at 45 °C with 5 units of S<sub>1</sub>. RNA from the microinjections of; lane 1, pX1Δ5'-1; lane 2, pX1ΔNS8 as analysed in **B** above. The diagrams indicate the positions (solid arrowheads) of nonhomology loops formed in hybrids between the wt probe and the deletion mutant transcripts, which are attacked by S<sub>1</sub> nuclease. Lanes 3-6, respectively, 0.01, 0.05, 0.25 and 1.25 μg *X. laevis* oocyte RNA plus in each case 25 μg *X. borealis* oocyte RNA.



**Fig. 5** The effects of spacer mutations on 40S transcription. **A**, The structure of the ribosomal DNA fragments in pX1108c (wt), pX1ΔES1 (ΔES1), pX1ΔNS8 (ΔNS8) and pX1ΔNS1 (ΔNS1). The construction and base sequence of these mutants were described previously<sup>10</sup>. The restriction sites used in their construction are indicated by: M, *Mbo*II; Ha, *Hae*II; B, *Bgl*I; H, *Hga*I. The 5'-[<sup>32</sup>P]-end-labelled probe used in **B** below is indicated (S<sub>1</sub> probe) as is the position of the <sup>32</sup>P label (solid circle). **B**, Total oocyte RNA was S<sub>1</sub> mapped as in Fig. 4C, using the probe shown in **A** above. In lanes 1-9, 250 pg of pX1108c or an equimolar amount of pX1ΔNS1 or pX1ΔNS8 were co-injected with varying amounts of pX1ΔES1 into *X. borealis* oocytes. Lanes 1-3, pX1108c and, respectively, 250, 25 or 2.5 pg of pX1ΔES1. Lanes 4-6, pX1ΔNS1 and, respectively, 250, 25 and 2.5 pg of pX1ΔES1. Lanes 7-9, pX1ΔNS8 and, respectively, 250, 25 or 2.5 pg pX1ΔES1. Lanes 10-13, respectively, 0.01, 0.05, 0.25, and 1.25 μg *X. laevis* oocyte RNA plus in each case 25 μg *X. borealis* oocyte RNA. The diagrams indicate the heteroduplexes formed between the probe and transcripts and the positions of the S<sub>1</sub>-protected fragments (arrows). The double band produced by the pX1ΔES1 transcript is due to S<sub>1</sub> attack just within the heteroduplex<sup>10</sup>. The transcription from pX1ΔES1 is therefore proportional to the sum of these bands.





transcription of the NTS *in vivo* and that this transcription is most probably terminated (not processed) at a site 213 ( $\pm$ 2) bp upstream of the 40S pre-rRNA initiation site. The results are consistent in all aspects with electron microscope observations of NTS transcription *in vivo*<sup>11-14,16</sup> and present for the first time the molecular mechanism of this transcription. Further, it has been shown that deletion of the *Bam* islands and associated sequences from the NTS strongly reduces the initiation of 40S pre-rRNA, when the gene is in competition with the wild type for transcription factors. More than 95% of the 40S transcription in such experiments came from the wild-type gene and little or no transcription was detected from the deletion mutant. Because RNA polymerase I clearly recognizes the *Bam* islands, it is extremely likely that such competition for transcription factors is at least in part due to the affinity of the *Bam* islands for polymerase. The NTS is then acting as a binding site or 'sink' for RNA polymerase I.

Deletion of the *Bam* islands reduces by two-thirds the total number of RNA polymerase I promoters on the gene studied here. This might then be expected to reduce the gene's affinity for polymerase by a similar factor. However, as mentioned above, experiments with *Bam* island deletion mutants showed that the affinity for transcription factors was apparently reduced by a factor of 20 or more. Thus, sequences in addition to the *Bam* islands may also be involved in polymerase binding. Results with the NTS deletion mutants pX $\Delta$ NS8 and pX $\Delta$ NS1 (Fig. 5) demonstrated that if sequences other than the *Bam* islands are involved, they must lie in repetitive region 2 and, by sequence homology, repetitive region 3 (Fig. 6). These regions consist of homologous and tandemly repeated 60- and 81-bp units (Fig. 6B). The sequence common to each unit is however, also homologous to the -100 bp region of the 40S promoter<sup>10</sup> (Fig. 6B) and is found in repetitive elements of both the *X. borealis* and *X. clivii* ribosomal spacers (ref. 8 and R. Clerc and A. Bird, personal communications). Furthermore, the demethylation of repetitive regions 2 and 3 of the *X. laevis* NTS correlates with the activation of the ribosomal

genes in early development<sup>20</sup>. It is therefore quite possible that repetitive regions 2 and 3 of the *X. laevis* NTS constitute repeated RNA polymerase I binding or entry sites. This latter possibility is suggested by the position in the 40S promoter of the sequence found duplicated in the repetitive regions (Fig. 6B).

Figure 6A presents a model of NTS function based on the above observations and discussion. RNA polymerase I from the nuclear pool binds either to the *Bam* islands or to repetitive regions 2 and 3. Polymerase molecules entering at the *Bam* islands initiate transcription and in transcribing the downstream repetitive regions, provide the driving force to move uninitiated polymerase, which it is speculated above could be bound there, towards the 40S promoter. Termination of NTS transcripts at -213 bp then makes all the polymerase molecules available for 40S initiation. Whether the polymerase molecules remain bound on the DNA or are released at the -213 bp termination site does not alter the overall effect of the spacer. In either case the concentration of polymerase at the 40S promoter would be much higher than that in the nuclear pool and hence 40S initiation would be stimulated or enhanced. Some experiments<sup>16</sup> (and readthrough on pX $\Delta$ NS1, Fig. 5) have even suggested that it may also be possible for polymerase molecules completing an upstream 40S pre-rRNA transcript to be captured by the following NTS (Fig. 6A) and hence transcribe several ribosomal genes in tandem.

It is interesting to draw a parallel between the 72-bp enhancer sequence of SV40<sup>21</sup> and the 60/81-bp sequences of the *X. laevis* ribosomal NTS, in that both may provide entry sites for RNA polymerase. We are presently studying the validity of this parallel.

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## LETTERS TO NATURE

### Active galaxies and the diffuse $\gamma$ -ray background

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A new model<sup>1</sup> for the origin of relativistic particles and  $\gamma$  rays in active galactic nuclei and quasars, together with recent HEAO 1 observations<sup>2</sup> of the spectra of active galaxies from 2 to 165 keV, provide the basis for the present re-examination of the nature of the extragalactic  $\gamma$ -ray background<sup>3,4</sup>. We report here that active galaxies could account for the observed background if their X-ray spectra steepen to  $E^{-2.1}$  above ~100 keV, as has been observed in Cen-A (ref. 5), together

with a further steepening to  $E^{-2.7}$  as a result of absorption of  $\gamma$  rays by photon-photon pair production interactions with X-ray photons. The compactness of active galaxies required to give this steepening is consistent with current estimates of their typical luminosity and radius.

The observed spectrum<sup>6</sup> is shown in Fig. 1. These recent observations are consistent with results reviewed earlier<sup>7-9</sup> and in particular the feature at MeV energies observed in balloon experiments (see ref. 8) is also apparent in the more recent data. The X-ray spectrum from 3 to 50 keV is consistent with thermal bremsstrahlung from a plasma at ~40 keV and may result from a hot intergalactic medium<sup>10</sup>, while at the highest energies (30-150 MeV) the  $\gamma$ -ray photon spectrum is consistent with a power law of index 2.7. Several other possibilities have

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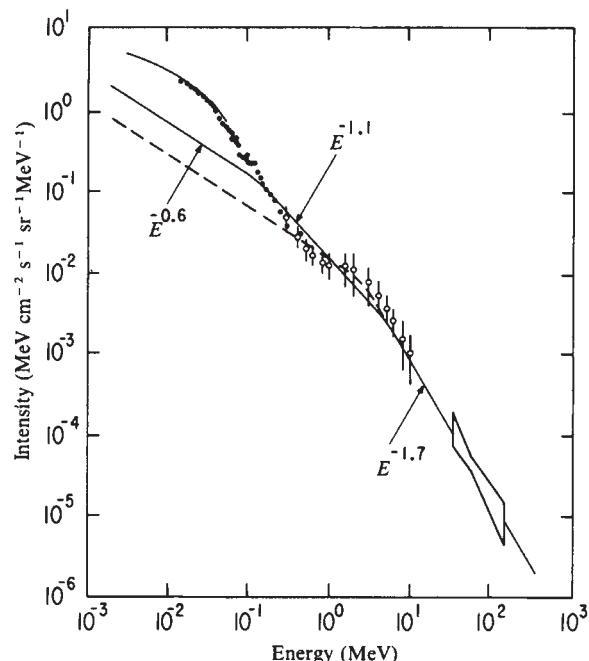
been proposed to account for the observed diffuse  $\gamma$ -radiation (see refs 7, 11, 12). Stecker<sup>13</sup>, for example, has suggested that it may result from matter-antimatter annihilation in a baryon symmetric universe, the annihilation occurring at the boundaries between domains of matter and antimatter. Emission from unresolved active galaxies has also been proposed to account for the diffuse  $\gamma$ -ray flux<sup>14-17</sup>. To date, only one extragalactic object, the quasar 3C273, has been detected at 100-MeV energies<sup>18</sup>. The contribution of quasars to the  $\gamma$ -ray background is, however, uncertain as the volume density of  $\gamma$ -ray emitting quasars is unknown. Seyfert 1 galaxies show flat power law energy spectra at X-ray energies<sup>19</sup> and could contribute significantly to the diffuse  $\gamma$ -ray flux. We consider the latter possibility below.

Recent HEAO 1 observations by Rothschild *et al.*<sup>2</sup> of 12 active galaxies (the sample is dominated by Seyfert 1 galaxies) show that the X-ray photon spectra of all these objects from 2 to 165 keV are consistent with a single power law with exponent  $\sim 1.6$ . Upper limits exist above 30 MeV from SAS 2 observations of seven of these objects<sup>17</sup> and indicate that their spectra must steepen between hard X-ray and  $\gamma$ -ray energies.

Rothschild *et al.*<sup>2</sup> also showed, using the luminosity function of active galaxies from the HEAO 1 flux limited X-ray survey<sup>20</sup>, that active galaxies could account for all of the diffuse X-ray emission above  $\sim 150$  keV with no evolution. It has also been suggested<sup>21</sup> that active galaxies might account for the observed flattening in the hard X-ray background from 500 keV to 2 MeV. We propose that the  $\gamma$ -ray background may also be due to these unresolved active galaxies and will show how the observed spectral shape could arise naturally in the model for relativistic particles and  $\gamma$ -rays in active galaxies and quasars described in our earlier work<sup>1</sup>.

The model<sup>1</sup> we use for the X-ray and  $\gamma$ -ray spectra of active galaxies has already been applied to the observed spectrum of 3C273. The salient features of this model are: (1) protons are accelerated by the first order Fermi mechanism at a shock in a spherical accretion flow onto a massive black hole; (2) relativistic protons have a spectrum which is a power law in energy  $E^{-(2+\epsilon)}$ , where  $\epsilon \ll 1$  for strong shocks; (3) nuclear interactions result in production of  $\gamma$  rays from  $\pi^0$  decay and electrons from  $\pi-\mu-e$  decay, both being produced with spectra  $E^{-(2+\epsilon)}$  above several hundred MeV; (4) synchrotron and inverse Compton losses steepen the ambient electron spectrum to  $E^{-(3+\epsilon)}$ ; (5)  $\gamma$ -ray photons from inverse Compton interactions then have a photon spectrum  $E^{-(2+\epsilon/2)}$ , very similar to that of  $\pi^0$   $\gamma$  rays; (6) the high-energy  $\gamma$ -ray spectrum is steepened by photon-photon pair production interactions with X rays.

In this model, one might initially expect the  $E^{-(2+\epsilon/2)}$   $\gamma$ -ray photon spectrum to continue without a break to X-ray energies, contrary to observation. A break in the electron spectrum from  $E^{-(3+\epsilon)}$  at high energies to  $\sim E^{-2}$  at low energies, may, however, manifest itself as a break in the photon spectrum from  $E^{-(2+\epsilon/2)}$  at  $\gamma$ -ray energies to  $\sim E^{-1.5}$  at X-ray energies. Two possibilities may account for this. The first is that relativistic particles might leak out of the central object on some time scale  $t(\text{leak})$ , perhaps producing the beams or jets associated with many active galaxies<sup>22</sup>. At energies below which the energy loss time is equal to the leakage time, that is  $E/(dE/dt) > t(\text{leak})$ , the ambient electron spectrum would have the same power law index as that at production, that is  $2+\epsilon$ . The power law index of the photon spectrum of inverse Compton X rays would then be  $(3+\epsilon)/2$ ; the observed spectral index of 1.6 implying  $\epsilon = 0.2$ , consistent with shock acceleration. The second possibility is that the observed X-ray spectral index may result from the kinematical cutoff of secondary electrons, that is those resulting from  $\pi-\mu-e$  decay. The production spectrum of secondary electrons<sup>1</sup> due to pion production cuts off steeply below  $\sim 100$  MeV below which knock-on electrons become important. There is thus a change in the production spectrum which may manifest itself as a change in slope of the inverse Compton radiation. In the case of no leakage (energy losses balance production) and if the knock-on component were small, the



**Fig. 1** Energy spectrum of the diffuse X-ray and  $\gamma$ -ray background (solid curve from 3 to 50 keV, ref. 7, ●, preliminary HEAO -1 data reported in ref. 2; ○, ref. 3; 30-150 MeV data, ref. 4). Solid and dashed lines show possible active galaxy contributions to the diffuse background (see text for discussion); a power law approximation to the energy spectrum is indicated for three energy regions. (For power-law spectra the energy spectrum is one unit flatter in the energy index than the photon spectrum; in the text photon rather than energy spectra are used.)

ambient spectrum would be inversely proportional to the rate of energy loss below the cutoff energy; that is an  $E^{-2}$  spectrum if synchrotron and inverse Compton losses dominate. The resulting X-ray photon spectral index would be 1.5, just slightly flatter than the observed spectrum. The inclusion of a knock-on component will, however, steepen the spectrum slightly.

In active galaxies, we expect a further steepening in their  $\gamma$ -ray spectra due to photon-photon pair production interactions with X-ray photons. For the photon spectrum which is observed at X-ray energies in most active galaxies,  $E^{-1.6}$ , the  $\gamma$ -ray spectral index would be steepened by 0.6, relative to that at production, above the energy at which the optical depth to this process is unity. This break energy depends on the X-ray source compactness, the ratio of X-ray luminosity to the dimensions of the emitting region. The expected steepening in photon spectral index from  $(2+\epsilon/2) = 2.1$  to 2.7 is just what is required if the observed diffuse  $\gamma$ -ray background is due to unresolved active galaxies.

The radio galaxy Cen-A has a very similar X-ray spectrum<sup>5</sup> to the active galaxies observed by Rothschild *et al.*<sup>2</sup>; indeed, the compact object responsible for the X-ray emission from Cen-A may be of the same class of object as the X-ray sources in Seyfert nuclei (the nucleus of Cen-A is obscured at optical to IR wavelengths by dust). In Cen-A, the spectrum is seen to break from  $E^{-1.6}$  below 140 keV to  $\sim E^{-2}$  above 140 keV (ref. 5). Such an  $E^{-2}$ , or slightly steeper, spectrum is expected if the relativistic particles result from shock acceleration and the emission is due to inverse Compton scattering of soft photons. A further steepening must then occur at some energy above a few MeV to be consistent with the SAS 2 upper limit<sup>18</sup>. This could then result from absorption of high-energy  $\gamma$  rays by photon-photon pair production interactions with the X-ray photons. (The observation of a flux of  $\gamma$  rays above 100 GeV from Cen-A<sup>23</sup> which is above an  $E^{-2.7}$  extrapolation from the SAS 2 upper limit merely implies, in this model, that the photon density at  $\sim 2.5$  eV in the region of the compact  $\gamma$ -ray source is low.)

Figure 1 shows (solid line) the expected contribution of active galaxies to the X-ray background calculated, assuming no evolution, by Rothschild *et al.*<sup>5</sup> from the X-ray luminosity function of active galaxies obtained by Piccinotti *et al.*<sup>20</sup> and taking a low luminosity cutoff at  $3.5 \times 10^{42} \text{ erg s}^{-1}$ . We have extrapolated this spectrum to 140 keV where we have assumed that the X-ray spectrum of average active galaxies smoothly breaks from  $E^{-1.6}$  to  $E^{-2.1}$ , in a way similar to that observed in Cen-A. A further break is then required at  $\sim 5 \text{ MeV}$  (to  $E^{-2.7}$ ) to account for the observed  $\gamma$ -ray background from 30–100 MeV (see Fig. 1).

We shall now calculate the X-ray source compactness required to account for the  $\gamma$ -ray background with unresolved active galaxies. We do not present here results of a full cosmological integration which would take into account the redshifting of energy spectra of distant active galaxies which contribute to the background. We have found, however, that the effect on the spectral shape is small in the present case, the greatest contribution to the  $\gamma$ -ray flux being due to relatively nearby active galaxies. The optical depth of  $\gamma$  rays of energy  $E_\gamma$  to photon–photon pair production is given by:

$$\tau(E_\gamma) = s \int n_x(E_x) \sigma(E_\gamma, E_x) dE_x$$

where:  $s = 1.33R$  is the average distance through the source, of radius  $R$ ;  $n_x(E_x)$  is the average density of X-ray photons at energy  $E_x$  and is given by

$$n_x(E_x) = L_x(E_x) E_x^{-2} / (\pi R^2 c)$$

where  $L_x(E_x)$  is the X-ray luminosity at energy  $E_x$  (energy per unit time per  $\ln[\text{energy}]$ );  $\sigma(E_\gamma, E_x)$  is the cross-section<sup>24</sup> for a  $\gamma$ -ray photon of energy  $E_\gamma$  producing a pair on interacting with an X-ray photon of energy  $E_x$ . For  $n_x(E_x) \propto E_x^{-1.6}$ , and an optical depth of unity at the required break energy,  $\tau(5 \text{ MeV}) = 1$ , we find for the X-ray source compactness,  $L_x(2\text{--}10 \text{ keV})/R = 6 \times 10^{27} \text{ erg s}^{-1} \text{ cm}^{-1}$ . For a luminosity function<sup>20</sup> proportional to (luminosity)<sup>-2.75</sup>, the mean luminosity is 2.4 times the low luminosity cut-off,  $L_1$ . Then, for  $L_1(2\text{--}10 \text{ keV}) = 3.5 \times 10^{42} \text{ erg s}^{-1}$  (solid line in Fig. 1), the mean luminosity is  $8.4 \times 10^{42} \text{ erg s}^{-1}$  and we obtain a typical source radius of  $1.4 \times 10^{15} \text{ cm}$ . The low luminosity cutoff in the X-ray luminosity function of active galaxies is, however, uncertain. If  $L_1$  were  $10^{43} \text{ erg s}^{-1}$ , then the contribution to the X-ray background<sup>2</sup> would be as shown by the dashed line in Fig. 1. The  $\gamma$ -ray observations would then permit a single spectral break from  $E^{-1.6}$  to  $E^{-2.7}$  at about 2.5 MeV (energy at break from spectral index of 1.6 to 2.1 coincident with that from 2.1 to 2.7), producing the observed flattening in the energy spectrum at MeV energies. On this assumption, the X-ray compactness would be  $9 \times 10^{27} \text{ erg s}^{-1} \text{ cm}^{-1}$  for a typical active galaxy, corresponding to a source radius of  $2.7 \times 10^{15} \text{ cm}$ . Values of the radii of Seyfert 1 galaxies obtained from their X-ray time variability<sup>25</sup> range from  $6 \times 10^{14}$  to  $8 \times 10^{16} \text{ cm}$  and bracket the values we obtain here.

If the bulk of the diffuse  $\gamma$ -ray background is due to active galaxies, one might expect considerable fluctuations in the diffuse flux to arise from the few nearest sources. Unfortunately, the magnitude of the fluctuations we expect, after appropriately scaling the corresponding fraction of the X-ray fluctuations due to active galaxies<sup>26</sup>, is well below the upper limits of the SAS 2 data<sup>4</sup>. Even the Gamma Ray Observatory (GRO)<sup>27</sup>, with its large area and long exposure time, may still be limited by photon statistics. If, however, the model proposed<sup>1</sup> for the  $\gamma$ -ray spectra of active galaxies is correct, GRO should be able to detect several of these objects. It will then be possible to infer the active galaxy contribution to the diffuse  $\gamma$ -ray background and thus provide a test for the present hypothesis.

We conclude that the observed spectrum of the diffuse  $\gamma$ -ray background could be accounted for by unresolved active galaxies, even with no evolution, if their spectra are described by a spherical accretion–shock acceleration model<sup>1</sup> and are

steepened by photon–photon pair production interactions at high energies.

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## Long-lived particulate or gaseous structure in Saturn's outer magnetosphere?

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During the encounters with Saturn by the Voyager 1, Voyager 2 and the Pioneer 11 spacecraft, large variations in the number density of low-energy plasma ions were observed in the outer magnetosphere. Those density variations were thought to be due to changing external conditions, remnants of gas from Titan, or detached portions of Saturn's main plasma sheet. We have found evidence which suggests that at least some of the density variations (which appear as dips in a curve which generally shows rising density as Saturn is approached) are due to absorbing material near Saturn's equatorial plane in the region between 10 and 20 Saturn radii ( $R_s$ ). Although the entire picture is still speculative, we present our evidence to encourage further optical examination of that region near Saturn. Notably, in 1978 Colombo suggested that a deep survey be made of the region between Tethys and Titan (10–20  $R_s$ ) because of its similarity to the asteroid belt in the Solar System<sup>1</sup>.

Figure 1 shows the flight paths of the three spacecraft. As depicted in Fig. 1a, Pioneer 11 and Voyager 1 approached at relatively low latitudes (on 1 September 1979 and 12 November 1980, respectively); Voyager 2 was generally at higher latitudes during its approach on 25 August 1981.

Since the low energy plasma is confined to the magnetic field lines from Saturn, we compare the low and high latitude observations by plotting them against  $L$ , a parameter introduced by McIlwain, which for a simple dipole saturnian field specifies the position (in  $R_s$ ) where the field line at the spacecraft passed through Saturn's equatorial plane. Figure 2 displays 15-min averages (covering  $\sim 0.25 R_s$ ) of plasma ion data from the two Voyagers<sup>2,3</sup> as well as every data point available from the Pioneer 11 plasma experiment<sup>4</sup>. Note that the Pioneer observations are divided by 10 for clarity; and that when adjusted upward by a factor of 10, the general behaviour of the Pioneer data agrees well with the Voyager 1 observations. The generally lower values of number density observed from the Voyager 2



at higher latitudes, can be understood in terms of a model in which the rotational motion of the magnetospheric plasma causes the heavier ion components to accumulate near the equatorial plane; that tendency is opposed by the ion and the electron thermal motion. Such a model has been used at Jupiter by, for example, Hill and Michel<sup>5</sup>.

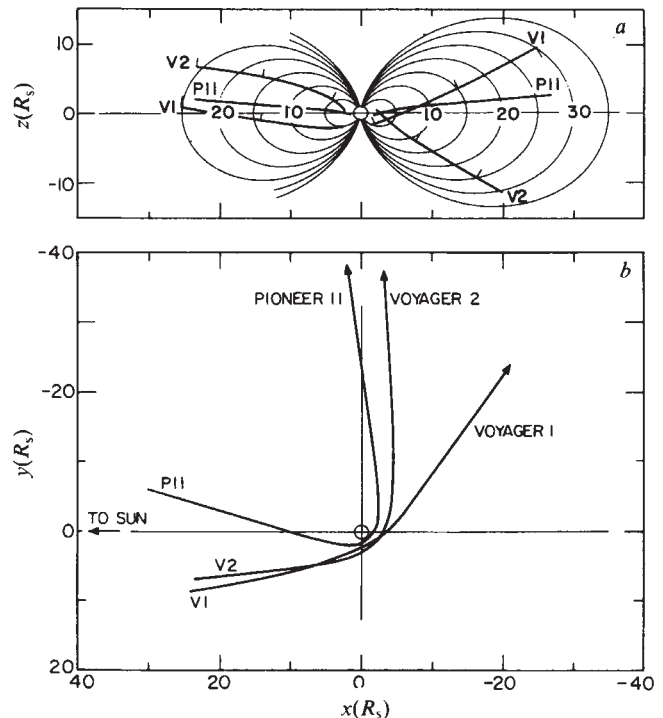
The plotted Voyager number densities were determined by taking the total particle flux density measured with a sensor that looked nearly into the direction expected for co-rotating magnetospheric flow and dividing by the velocity component into that sensor expected for rigidly co-rotating flow. More precisely, the ion current was divided by the sensor area, the unit charge, and the expected co-rotation velocity component. The resulting quantity is the number of elementary charges per unit volume. This procedure was used to bridge regions where higher ion temperatures made model fits difficult. Fits of convected maxwellian distributions to the cooler distributions and model fits to the observations of the electron component of the plasma both give results agreeing with those densities to ~10%. The electrons are subsonic and hence less dependent on specific flow velocities. Therefore, we conclude that the density variations reported here are not critically dependent on assumptions about the flow or the plasma thermal speed. The number density in the dip near  $L = 14$  is approximately at the instrument noise level on Voyager 2; the Voyager 1 noise level is lower than that of Voyager 2. The Pioneer densities were determined from fits assuming flow in the co-rotation direction but at less than the rigid co-rotation speeds.

As mentioned above, the data suggest that the general tendency for the ion number density to increase as Saturn is approached is interrupted by abrupt decreases. On the basis of observations from Voyager 1 and Voyager 2, the Voyager plasma team had been considering two models: the higher density regions could be detached patches of dense plasma from the nearer-to-Saturn plasma sheet centred about Saturn's equatorial plane or they might be related to crossing Titan-released plasma plumes that extend downstream in the plasma flow<sup>3</sup>. Structure observed near the same value of  $L$  but at different latitude and persisting over a period of 2 yr suggests, instead, the presence of one or more long-lived partial absorbers in the equatorial plane. When the finest time scale data are examined, the dip near  $L \approx 14$  extends from  $L = 13.7$  to 14.5 and another dip extends from  $L \approx 18.3$  to 19. Both dipoles appear to have edges sharp compared with their width. These large widths, of the order of 1 Saturn radius, suggest distributed absorbers since the gyroradius of the ions involved (for example, proton thermal speeds  $\approx 100 \text{ km s}^{-1}$ ) is only of the order of 100 km. If the source of plasma were only Titan or closer to Saturn, the density after a dip would not recover to its earlier value. Observations thus suggest that there is more than one source.

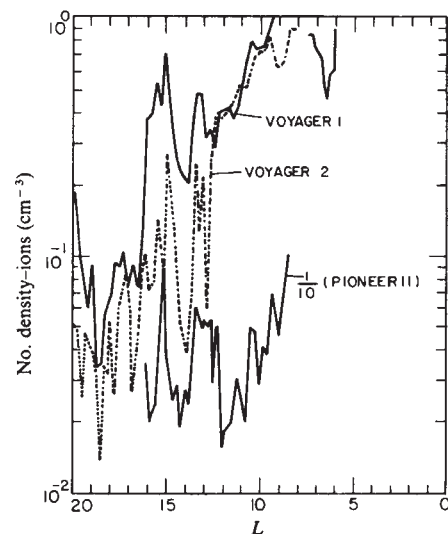
Considerable structure near  $L = 14$  is also seen in data from other Voyager experiments. The cosmic ray subsystem (CRS) data<sup>6,7</sup> (0.14–0.4 MeV electrons) shows structure and a significant dip near  $L \approx 14$  incoming on Voyager 1 and 2. The Voyager 1 UV spectrometer data<sup>8</sup> show an increase in the scattered Ly- $\alpha$  data just inside  $15 R_s$ , through no obvious structure is seen in the Voyager 2 UV data<sup>9</sup>.

The outgoing observations need further examination. The low-energy ion flow is supersonic and none of the plasma ion experiments was oriented so that it could observe magnetospheric ions in the outer magnetosphere as the spacecraft left Saturn. On the other hand, the plasma electrons are subsonic and that portion of the plasma experiment should have been able to see structure in the outgoing leg of the trajectory. Structure is seen in the plasma electron<sup>2</sup> and CRS data<sup>5</sup> in the outer magnetosphere from Voyager 1, though no structure is seen from Voyager 2. Coping with the distorted magnetic field expected in the tail-like region traversed by Voyager 1 suggests that these data be studied in the future.

There is already a problem concerning the use of a simple dipole model to compare higher latitude Voyager 2 data with



**Fig. 1** Spacecraft trajectories: *a*, the trajectories rotated about Saturn's spin and (coincident) dipole axis onto a common meridian plane; *b*, the trajectories projected onto Saturn's equatorial plane. The  $z$ -axis coincides with Saturn's spin axis, the  $x$ -axis is the projection of the Saturn-Sun line onto the equatorial plane, and the  $y$ -axis completes the right-handed coordinate system.



**Fig. 2** Ion number densities observed in Saturn's outer magnetosphere from three incoming spacecraft spanning 2 yr. The abscissa is the magnetic shell parameter for a simple dipole field. The Pioneer 11 data have been reduced by a factor of 10 for clarity.

the nearer equatorial Voyager 1 and Pioneer observations. In fitting the Voyager magnetometer observations, Connerney *et al.*<sup>10</sup> found it necessary to add an equatorial ring current extending from  $\sim 10$  to  $15 R_s$ . The resulting distortion of the field line shape is most significant near the dip plotted at  $L = 14$ ; the Voyager 2 dip would be moved outwards approximately  $1 R_s$ , if their field model were used thus weakening the correlation, though the correlation between Voyager 1 and Pioneer 11 seems remarkable in itself. (The Voyager 2 data would be shifted seriously only near  $L = 14$ .) Connerney *et al.* also point out that the field observed from Voyager 2 in that region is not what one would expect since its direction was rotated

towards the Sun, perhaps due to a decrease in the external solar wind pressure. We must conclude that the extrapolation process for Voyager 2 is not well defined in that region and the general agreement throughout the passes seems to be better than could be explained by chance. The situation remains speculative but warrants examination by other means.

Columbo pointed out that the positions of the maxima surrounding the  $L \approx 14$  dip could represent regions where the equatorial absorbing material has been cleared out by resonances with Titan's orbital period (3:2 at  $15.5 R_s$  and 2:1 at  $12.8 R_s$ ).

We conclude that the plasma experiments on Voyager 1 and 2 and on Pioneer 11 show outer magnetospheric structure at the same position in  $L$ . The observations suggest the presence of absorbers in the equatorial plane whose nature may be debris or possibly gas. It would be interesting to see if the presence of this material could be detected by direct observations of scattered light or by stellar occultation experiments.

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## An interpretation of the dawn–dusk asymmetry of UV emission from the Io plasma torus

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Recently, the Voyager UVS experimenters made two interesting reports on the emission morphology and energetics of the Io plasma torus. First, Sandel and Broadfoot<sup>1</sup> provided evidence for a localized energy source in the vicinity of Io with the time-averaged power amounting to 20% of the total power radiated by the Io UV torus. Second, Shemansky and Sandel<sup>2</sup> demonstrated that the Io torus displays a local time variation in the extreme UV (EUV) emissions with a dawn-to-dusk asymmetry differing by ~30% and that the corresponding electron energy density available for exciting the UV emissions of the oxygen and sulphur ions varies by ~20%. These observations provide an important insight into the mass and energy flows of the Io plasma, but call into question our understanding of the physical mechanisms that maintain the radiative emissions of the heavy ions ejected from Io. Here we discuss the physical cause of the brightening near dusk side and dimming near dawn side of the Io plasma torus EUV emissions in terms of a drift shell effect. If the radial distance of the  $L$  shell of the Io plasma torus reaches a minimum around 1800 h LT but reaches a maximum around 0600 h LT, periodic adiabatic heating and cooling of the electron gas could lead to the observed modulation of the UV irradiation. Such a drift pattern of the Io torus could be produced if the plasma convection of the jovian magnetosphere were partly determined by its planetary wind outflow.

After the Voyager observations of Jupiter, the major issue concerns how to relate the auroral emissions near the footpoints

of the Io torus to the UV emissions of the Io torus itself. An apparent consensus<sup>3–5</sup> is that the auroral emission is caused by the atmospheric precipitation of energetic protons and heavy ions which in turn produces a flow of secondary electrons beaming back into the Io plasma torus. These secondary electrons of energies  $\geq 10$ –20 eV could provide the required heat source for the torus electrons if the precipitating flux of heavy ions having energies  $\leq 500$  keV is intense enough<sup>5</sup>. Other electron acceleration mechanisms could also be important. For example, the energy transfer from the new ions created by charge exchange between the co-rotating thermal ions and the neutral atoms<sup>6–8</sup> to the electrons could be significant<sup>9–11</sup>. This might mean that there exists strong interaction between the electrons and electrostatic or electromagnetic plasma waves even though these effects have not been well established by observations.

By the same token, wave-particle scattering and electrostatic acceleration processes in the vicinity of the Io flux tube might be particularly strong to enhance the atmospheric precipitation of energetically charged particles as well as injection of suprathermal electrons into the co-rotation wake of Io. Note that the total current of  $3 \times 10^6$  A flowing along the Io flux tube<sup>12</sup> implies a power of  $2 \times 10^{11}$  W as a result of the unipolar induction mechanism of Io in the jovian magnetosphere. If this current is carried mainly by suprathermal electrons<sup>13,14</sup>, local dissipation of this power would lead to the Io-related hotspot of UV emissions as investigated by Sandel and Broadfoot<sup>1</sup>. The local time variation of the Io torus emissions<sup>2</sup>, however, could not be interpreted in this manner, because precipitation of energetic particles by pitch-angle scattering into loss cone presumably extends throughout the whole torus without longitudinal restriction<sup>15–17</sup>. This view is partially supported by the Voyager observations of the continuous distribution of the auroral emissions magnetically connected to the Io torus<sup>10,18</sup>. There is difficulty, therefore, in relating the secondary electron heat flow effect to the conclusion<sup>2</sup> that the dawn–dusk asymmetry is caused by a maximum of energy injection into the Io torus at local noon.

One possible alternative is to assume that the energy injection rate into the Io plasma torus is approximately the same for all local times but that the excitation rate of the UV emissions is maximized at the dusk side and minimized at the dawn side. This could be achieved if the drift shell of the Io plasma torus has varying  $L$  values for different local times as periodic adiabatic expansion and compression of the flux tubes would allow the energy flux to the UV irradiation to be modulated in the desired direction.

In an attempt to invoke soft electrons as a heat source for Jupiter's thermosphere, Hunten and Dessler<sup>19</sup> have made the interesting point that inward convection of a magnetic flux tube containing  $N$  electrons from a radial distance  $L_1$  to  $L_2$  leads to the enhancement of the corresponding omnidirectional energy flux ( $Q$ ) by a factor of  $(L_1/L_2)^8$ . Briefly, the volume  $V$  of a magnetic flux in a dipole field varies as  $L^4$ :  $V \propto L^4$ . If the ratio of specific heats  $\gamma = 5/3$ , the electron thermal energy  $\epsilon$  can be given as:

$$\epsilon \propto V^{1-\gamma} \propto L^{-8/3} \quad (1)$$

Now, for  $Q = n u \epsilon$  where the particle velocity  $u \propto \epsilon^{1/2}$  and  $n = N/V$  we find directly

$$Q_2 = Q_1 (L_1/L_2)^8 \quad (2)$$

Thus for  $Q_2$  to be larger than  $Q_1$  by ~20%, as indicated by the UV observations of the Io plasma torus<sup>2</sup>, we need  $L_1/L_2 \sim 1.02$  or the radial distances at the dusk side (smaller) and the dawn side (larger) to vary by  $\sim \pm 1\%$ . To produce such a shift of the drift shell of the Io plasma torus, an electric field ( $E_i$ ) directed from dawn to dusk superimposed on the co-rotation electric field ( $E_c$ ) is required. For a magnetic field of ~2,000 nT and a co-rotation speed of 80 km s<sup>-1</sup>, the co-rotation electric field at Io's orbit is 0.16 V m<sup>-1</sup>. The magnitude of this cross-tail electric field therefore should be ~1.6 mV m<sup>-1</sup>.



As discussed by Goertz and Ip<sup>20</sup>, an electrostatic field of such nature can be established by a magnetosphere convection system driven by the jovian planetary wind or outflow of heavy ions emitted from Io. The initial assessment by Brice and Ioannidis<sup>21</sup> is that, because of its rapid rotation and strong dipole field, the jovian magnetosphere has predominantly the configuration of a co-rotating plasmasphere. (This theoretical idea has been qualitatively confirmed by the Voyager plasma experiments<sup>22-24</sup>.) Besides such a co-rotation convection system, Brice and Ioannidis<sup>21</sup> also discussed the presence of a plasma flow in the magnetotail directed towards the planet, driven by the solar wind interaction<sup>25</sup>. In this case, however, the resulting cross-tail electric field would point from dusk to dawn, and would produce a dawn-dusk asymmetry of the UV emissions in the Io plasma torus that was the opposite of the observations. The existence of a planetary wind outflow based on theoretical considerations<sup>26,27</sup> and Voyager observations<sup>24</sup> introduces the possibility that the actual cross-tail electric field could be reversed, producing the required Io plasma torus drift shell displacement.

Note that, under the assumption of uniform energy injection rate along Io's orbit, the idea presented here is not invalidated by the non-dipole nature of the jovian magnetic field<sup>28,29</sup>. This is because the thermal ions under consideration basically co-rotate with the magnetic field; without an externally applied electric field, there will be no change in the field strength at different local times. Thus, the only effect of periodic adiabatic heating-cooling must originate from the dawn-to-dusk electric field.

Thus, the interpretation of the local time variation in the UV emissions of the Io plasma torus must be constrained by the thermodynamic and electrodynamic couplings of the Io torus to the corresponding auroral zone; circumstantial evidence seems to exclude any drastic variation of the energy injection rate into the Io plasma torus between local noon and local midnight. In these conditions, a possible solution is to invoke the periodic heating and cooling of the electron gas in the Io plasma torus as a result of a slightly eccentric dipole drift shell. The proper modulation of the UV excitation rate can be obtained by the presence of an electrostatic field of  $\sim 1.6 \text{ mV m}^{-1}$  pointing from dawn to dusk. Such an electric field pattern is expected if the plasma convection in the jovian magnetosphere is determined by the outflow of its planetary wind. Conversely, the UV observations reported by Shemansky and Sandel<sup>2</sup>, in principle, can be diagnostic of the magnetospheric convection system.

After completion of this manuscript, we learned that Barbosa and Kivelson<sup>30</sup> have independently reached a similar conclusion on the physical cause of the dawn-dusk asymmetry of the Io plasma torus UV emission.

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## Beginning and end of lunar mare volcanism

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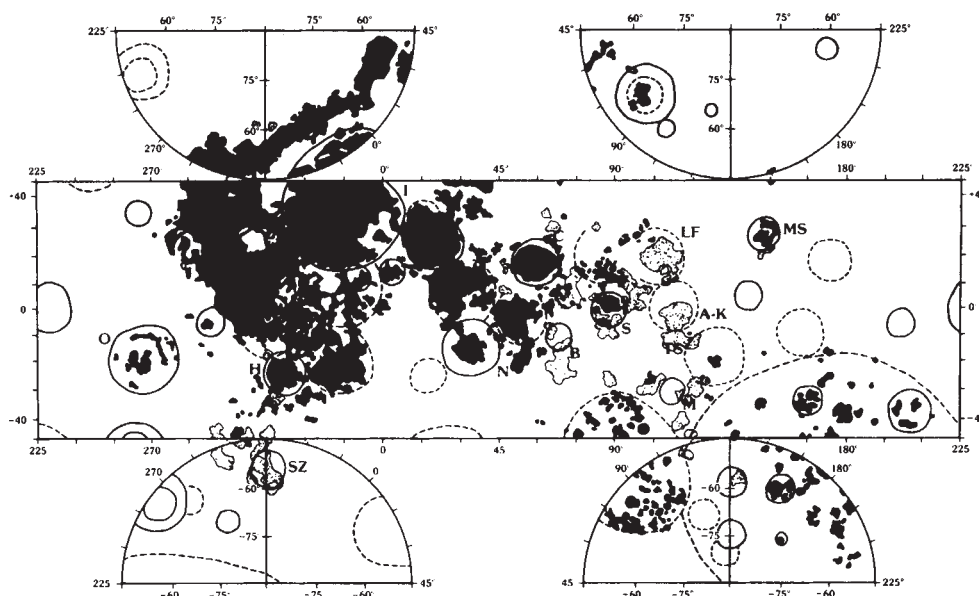
Mare volcanism on the Moon is commonly attributed to an important but relatively short-lived epoch of internal heating after 3,900 Myr BP but before about 2,500 Myr BP (refs 1, 2). Although some studies suggested that mare volcanism had started earlier<sup>3-5</sup> than times indicated by dated lunar samples, only recently have photogeological, spectral, and geochemical data<sup>6-8</sup> documented the importance of a pre-4,000 Myr BP epoch. Similarly, early Earth-based geological mapping revealed a bright-rayed crater (Lichtenberg) superposed by mare units<sup>9</sup>, thereby indicating that mare volcanism must have extended to times significantly more recent than 3,100 Myr BP. Lunar orbital photography confirmed this inferred stratigraphical relationship<sup>10</sup>, but crater statistics indicated that the youngest units were at least twice the age of Copernicus, or between 1,700 and 2,000 Myr BP (refs 11-13). We present here the inferred distribution and style of the early phases of mare volcanism based on current evidence and conclude that certain regions of the Moon underwent two distinct pulses of igneous activity. We then examine crater statistics for the post-Lichtenberg mare unit and other selected units and conclude that mare volcanism extended to a time comparable with that of the Copernicus impact, or  $\sim 1$  Myr BP. These reassessments of the oldest and youngest maria provide new constraints on geophysical models of the internal thermal history of the Moon.

Direct evidence for the existence of ancient lunar volcanism is found in a variety of sample investigations. Numerous mare basalt fragments are found as clasts in breccias that were assembled before 3,900 Myr BP (refs 14, 15). Many of these breccias appear to be related to major basins, suggesting that volcanic flows constituted at least part of the target for these impacts. Such volcanics could have existed either as lava flows covering the basin impact site or subsurface magma reservoirs ejected and subsequently included in basin deposits. Mixing model studies of highland soils (see ref. 16) require substantial admixture of a mare basalt component at a level significantly higher than that expected from post-mare lateral mixing by impacts<sup>17</sup>. This suggests the presence of a mare component within the highlands, either buried by and included within highlands megaregolith or deposited as basin ejecta.

The very early stages of mare volcanism are believed to be indicated by dark-haloed impact craters which have excavated ancient mare units from below light-coloured ejecta deposits<sup>6</sup>. More than 100 such craters larger than 1 km in diameter have been identified on the Moon. Apollo orbital geochemical data<sup>6,8</sup> and recent Earth-based spectral data<sup>7</sup> confirm the proposed mafic component in these ejecta. The distribution of dark-haloed impact craters can provide a basis for mapping buried mare basalt plains in order to understand the areal extent and the source regions. Figure 1 illustrates a conservative estimate

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**Fig. 1** Distribution of mare plains younger than 3,900 Myr (black) and buried mare plains (stippled) inferred from the clustering of dark-haloed impact craters and orbital geochemical data. Buried mare units are probably more widespread but their signature has been masked through vertical and lateral mixing by impact cratering including the formation of the large multi-ringed basins. Such ancient mare surfaces are localized within very old impact basins in patterns similar to younger mare plains. Abbreviations represent the following selected multi-ring basins (after refs 4, 18, and 38): O, Orientale; H, Humorum; SZ, Schiller-Zucchi; I, Imbrium; N, Nectaris; C, Crisium; B, Balmer; S, Smythii; LF, Lomonosov-Fleming; AK, Al-Khwarizmi-King; TS, Tsiolkovsky-Stark; M, Milne; MS, Moscoviense.

of ancient mare deposits and reveals a close association with very old impact basins identified in refs 4 and 18. Although early analysis<sup>4</sup> of old impact basins led to the suggestion that such structures may have been filled with dark lavas whose surface lightened with age, dark-haloed impact craters were not cited as specific indicators of the nature and extent of this early phase of volcanism. The most extensive deposits are found in the eastern hemisphere: within the heavily degraded basin Lomonosov-Fleming and partly encircling the Smythii, Crisium, Milne, and Balmer basins. Additionally, dark-haloed craters indicate buried mare surfaces around the Schiller-Zucchi basin where thermal IR maps<sup>19</sup> clearly indicate a relative excess of blocks comparable with that of more recent unblanketed mare surfaces and in contrast with adjacent highlands.

The apparent absence of buried mare surfaces on the nearside (near 0°) and western hemispheres (270°) is believed to reflect masking by Imbrium and Orientale ejecta deposits. Although dark-haloed impact craters and geochemical signatures in these regions may indicate buried maria<sup>6</sup>, their occurrences are too isolated for defining significant contiguous plains. Poor photographic coverage may account for the absence of inferred buried maria between 180° and 270° and in the polar regions. Nevertheless, the total estimated areal extent of these ancient mare deposits exceeds  $7.5 \times 10^5 \text{ km}^2$  or ~12% of the visible-Imbrian and younger maria. This value should be viewed as a minimum since ponded lavas within craters and thin flows are difficult to recognize. Moreover, recent identification of a mafic anomaly in the region of the crater Firsov (4° N, 112° E) suggests the presence of a mare volcanic flow whose surface expression

**Table 1** Crater densities for young maria and absolutely dated surfaces

| Region                                | Lat. Long.       | Photograph no. | $D_L^*$<br>(km) | $N_1/A_1$<br>( $10^{-2}$ ) | $A_1$<br>( $\text{km}^2$ ) | Ref. | $N_2/A_2$<br>( $10^{-2}$ ) | $A_2$<br>( $\text{km}^2$ ) | Ref.   | Absolute†<br>age (Myr) | Ref. |
|---------------------------------------|------------------|----------------|-----------------|----------------------------|----------------------------|------|----------------------------|----------------------------|--------|------------------------|------|
| Apollo 15                             | 15.3° N, 2.8° E  | LO-V-105-M     | 270 ± 15        | —                          | —                          | —    | 5.0 ± 1.0                  | 373                        | 28, 39 | 3,290 ± 50             | 29   |
| Apollo 12                             | 3.1° S, 23.2° W  | LO-III-154-M   | 210 ± 20        | 4.5 ± 0.6                  | 1,372                      | —    | 4.8 ± 1.0                  | 351                        | —      | 3,170 ± 60             | 29   |
| Area B(AB)‡                           | 28.5° N, 58.5° W | A15M-1699      | 165 ± 25        | 3.0 ± 0.4                  | 1,735                      | —    | 3.3 ± 0.7                  | 695                        | —      | (2,000 ± 300)          | —    |
| Late Imbrium<br>Flows (LIF)           | 32.5° N, 22° W   | LO-V-160-M     | 165 ± 25        | 2.6 ± 0.5                  | 915                        | —    | 2.8 ± 0.9                  | 352                        | —      | (1,800 ± 300)          | —    |
| Mare Smythii<br>(MS)                  | 1.3° N, 91° E    | LO-I-15-M      | —               | 2.3 ± 0.4                  | 1,332                      | —    | 1.9 ± 0.5                  | 625                        | —      | (1,200 ± 400)          | —    |
| South-west of<br>(Maestlin<br>(SWM)   | 1.8° N, 41.7° W  | LO-II-201-M    | 165 ± 25        | 2.7 ± 0.5                  | 1,243                      | 39   | 1.8 ± 0.7                  | 392                        | —      | (1,200 ± 500)          | —    |
| Letronne mare<br>(LM)                 | 11° S, 43.5° W   | A16M-2995      | 225 ± 14        | —                          | —                          | —    | 1.8 ± 0.6                  | 400                        | —      | (1,100 ± 500)          | —    |
| Gruithuisen<br>mare (GM)              | 37° N, 43° W     | LO-V-185-M     | 225 ± 15        | 2.3 ± 0.3                  | 2,512                      | —    | 1.8 ± 0.6                  | 990                        | 39     | (1,100 ± 500)          | —    |
| Flamsteed Ring<br>mare (FRM)          | 2.21° S, 44.2° W | LO-III-187-M   | 165 ± 25        | 2.3 ± 0.4                  | 1,770                      | 39   | 1.4 ± 0.4                  | 663                        | —      | (900 ± 400)            | —    |
| South-east of<br>Lichtenberg<br>(SEL) | 31° N, 66° W     | A15P-366-370   | 165 ± 25        | 2.0 ± 0.2                  | 4,950                      | —    | 1.5 ± 0.3                  | 2,134                      | —      | (900 ± 300)            | —    |
| Copernicus<br>(ejecta)                | 7° N, 18° W      | LO-V-149-M     | 100 ± 12        | 1.4 ± 0.1§                 | 6,800                      | —    | 1.6 ± 0.3                  | 2,140                      | 39     | 850 ± 100              | 26   |
| Tycho (ejecta)                        | 24.5° N, 47.3° W | LO-V-201-M     | —               | 0.44 ± 0.1§                | 4,347                      | 40   | —                          | —                          | —      | 96 ± 5                 | 30   |

$N/A$  represents the number of craters larger than 0.5 km in diameter per  $\text{km}^2$ . Subscripts refer to nested counting areas. Large areas (subscript 1) consistently result in counts larger than counts for small areas (subscript 2) for very young surfaces but comparable counts for old surfaces owing to the effect of secondary impact clusters.

\*  $D_L$  is a limiting crater diameter for degradation as described in ref. 11.

† Absolute ages in parentheses indicate ages inferred from Fig. 3.

‡ Region used in ref. 13.

§ Values will be slightly larger than values in mare owing to target strength differences<sup>41</sup>.



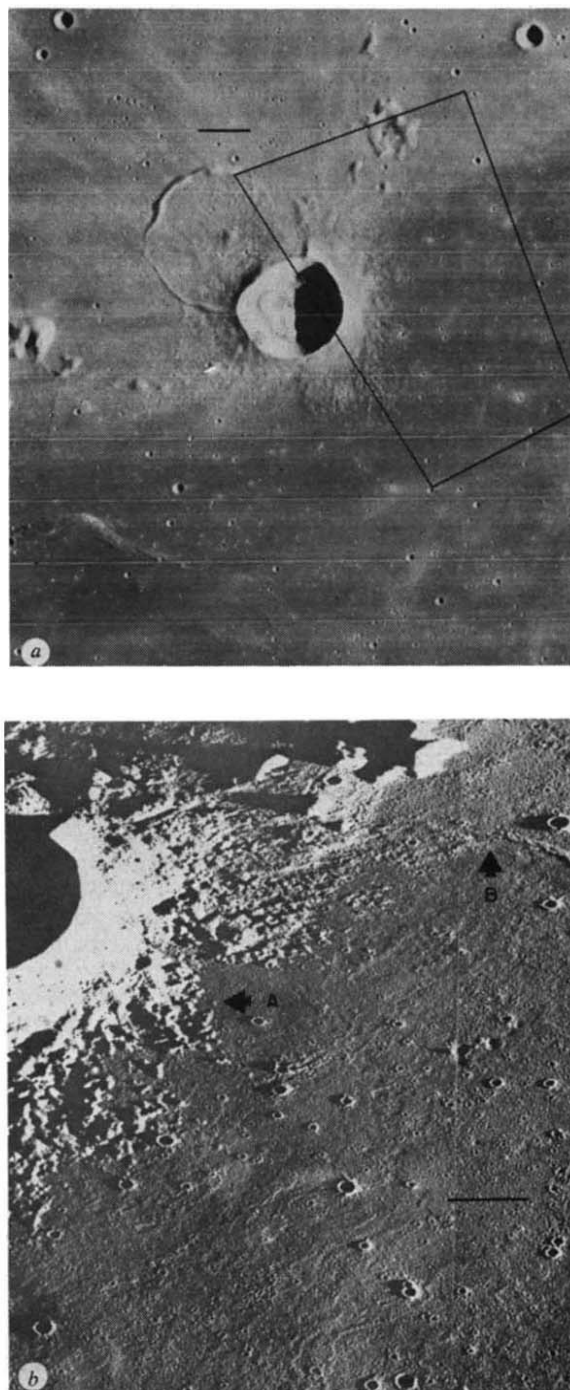
has been totally obliterated by saturation impact cratering within the proposed Al-Khwarizmi-King basin<sup>20</sup>. Thus numerous farside basins that appear to have no mare fill on the basis of albedo or surface features, may have undergone early mare flooding. The high cratering rates during the pre-Nectarian and Nectarian periods gardened and masked these units with the highlands megaregolith.

Figure 1 shows that several impact basins have undergone at least two separate stages of volcanic activity. Dark-haloed craters near Mare Smythii occur in smooth plains-filled craters within a very ill defined outer ring outside the principal boundary scarp containing floor-fractured craters and mare units. Crater statistics (see ref. 12) indicate that mare units in the interior of Smythii are very young, yet the buried, crater-contained units peripheral to the inner ring are very old. Apollo orbital geochemical data<sup>6,8</sup> support the interpretation that these peripheral units are volcanic in origin. Similarly, dark-haloed craters peripheral to Mare Crisium occur in low-lying concentric valleys with inferred ages much older than the dark interior mare plains. Recent high spatial resolution Mg/Al maps of this region reveal relatively high Mg/Al values in certain areas, some of which correlate with the occurrence of small dark-haloed craters<sup>21</sup>.

Certain models<sup>22</sup> suggest that late-stage volcanic activity occurred principally near the edge of the mare basins owing to tensional stresses from the load of the volcanic fill. The observations here do not negate this basic idea, but indicate in addition that the basin may undergo a period of resurgent activity concentrated along an interior ring<sup>4,23,24</sup>. Such volcano-tectonic resurgence can re-establish a battered circular outline (for example, for Smythii and Humorum) corresponding to deep-seated, impact-related fractures. Thus, concentric pools of lava (as inferred from dark-haloed craters and geochemical signatures) outside the principal rings of Smythii and Crisium may be remnants of a very early stage of volcanism. Such a two-stage model of mare flooding may reflect the relatively rapid isostatic adjustment of impact basins formed before ~4,000 Myr BP. As the lithosphere gained strength, deep-seated fractures along the inner rings of certain old basins provided conduits for later mare basalts. The subsequent outward migration of vents to the outer rings, however, may have ceased due to insufficient reloading of the basin interior, more limited magma reservoirs or deepening of the magma-source regions.

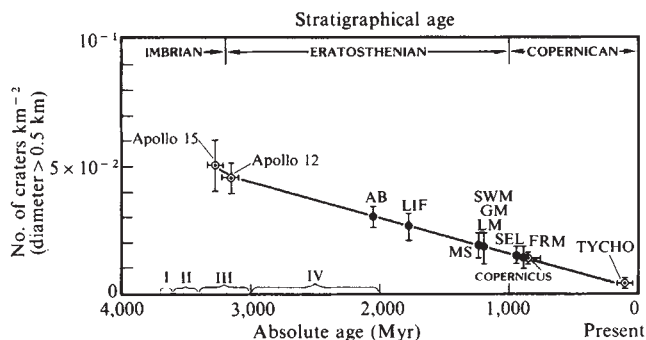
At the other end of the lunar time scale, current interpretations date the last stages of widespread lava flooding ~2,500 ± 500 Myr BP (ref. 25) with the youngest eruptions occurring around 1,700–2,000 Myr BP (refs 11, 13), about twice the estimated age of Copernicus<sup>26</sup>. However, the young mare units superposing the Lichtenberg ray system (Fig. 2) poses a contradiction in such interpretations. Bright-ray patterns from impact craters gradually disappear with time at a rate that increases with decreasing crater size<sup>27</sup>. Correlations between crater statistics and the rate of crater ray removal<sup>28</sup> indicate that a crater the size of Lichtenberg (20 km in diameter) would lose its bright rays if it were older than Copernicus (100 km). If Copernicus was formed at about 900 Myr BP (ref. 26), then the flow unit superposing Lichtenberg should have erupted at a similar time.

Crater statistics support the very young age of the basalt flows east of Lichtenberg. Figure 3 and Table 1 permit comparison of the number of craters larger than 0.5 km in diameter found on this unit that can be calibrated with lunar sample ages from Apollo 12 and Apollo 15 (ref. 29) and the inferred absolute ages of Copernicus<sup>26</sup> and Tycho<sup>28,30</sup>. The reference crater size of 0.5 km is large enough to avoid irregularities observed for crater statistics at small sizes<sup>31</sup> but small enough to obtain meaningful statistics for small counting areas. The number of superposed craters on the flows overlapping Lichtenberg approximates values for Copernicus. These statistics are based on enhanced near-terminator Apollo panoramic photographs that probably produce an overestimated age based on low-Sun corrections noted in (ref. 32). For comparison, Figure 3 shows the four mare age classes in (ref. 12) that extend to



**Fig. 2** *a*, The bright-rayed crater Lichtenberg (31.5° N, 67° W) in Oceanus Procellarum, embayed by younger mare units. The rate of crater ray removal for a crater of this size and the crater frequency on the mare units indicate a time of about 900 ± 300 Myr for mare eruption. The box corresponds to the area shown in *b*. Scale bar, 10 km. Lunar Orbiter IV-170-H1. *b*, A low-Sun, high-resolution view near Lichtenberg where mare units have embayed the ejecta deposits (arrow A) and covered the bright ray pattern. Arrow B identifies the contact between this young mare unit and older (but still relatively young) mare units of Oceanus Procellarum. Scale bar, 5 km. Apollo 15 Panoramic Photos 369 and 370.

~2,000 Myr BP. The youngest units previously cited include a region 400 km south-east of Lichtenberg whose inferred age is 1,800 ± 200 Myr BP (ref. 13) and the late Imbrium flows whose inferred age is 1,700 Myr BP. Our inferred age estimates are in agreement with previous studies, but in addition, we find several regions in Oceanus Procellarum having significantly younger ages. Moreover, very young mare flows are not



**Fig. 3** Number density of craters larger than 0.5 km on selected units versus absolute age as calibrated by four dated lunar events (circled). Actual values plotted are given in Table 1. Note pronounced cluster of inferred ages for mare units in the late Eratosthenian and early Copernican where time stratigraphical boundaries (top of graph) have been determined in ref. 38. Age groupings (I, II, III, and IV) at the bottom of the graph are from ref. 12. Abbreviations for selected mare units are given in Table 1.

confined strictly to the Oceanus Procellarum region as is commonly cited (see, for example, ref. 33). Table 1 and Figure 3 show that Mare Smythii is only slightly older than a large cluster of young maria in the Procellarum region (uppermost Eratosthenian). Older ages for the same units cited in ref. 12 reflect the use of techniques that integrate statistics from broad areas and different horizons, thereby combining and overestimating the age of the youngest units. Table 1 shows that statistics for craters  $>0.5$  km in similarly broad regions ( $>10^3$  km<sup>2</sup>) result in inferred relative ages consistent with those derived in ref. 12.

We suggest, therefore, that the end of mare volcanism extended to around the time of Copernicus or  $\sim 1,000$  Myr BP. The total areal extent of these last gasps of mare volcanism is impossible to determine from available image resolution. The very young units indicated in Fig. 3 and Table 1 all have retained very fine-scale (20–30 m) flow textures characteristic of thin lavas<sup>34</sup> and appear to correlate with spectrally distinct blue mare units in Oceanus Procellarum<sup>35</sup>. Such units cover a combined area of the order of  $10^4$  km<sup>2</sup>.

We conclude that lunar volcanism has been a relatively continuous process beginning perhaps as early as 4,300 Myr (the time when more basalt source regions became isotopically closed<sup>33</sup>) and gradually terminating around 1,000 Myr. The beginning of mare flooding was concentrated in old impact basins much in the style of post 4,000 Myr volcanism as suggested in ref. 1. Igneous activity ceased in numerous basins, perhaps reflecting isostatic adjustment during early lunar history; however, volcano-tectonic resurgence occurred within the inner rings of several basins well after 4,000 Myr. Volcanism extended to 1,000 Myr with possible minor eruptions postdating the Copernicus impact. This revised scenario is more consistent with theoretical thermal models<sup>36,37</sup> where the lunar interior gradually cools without a sudden termination.

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## Comparison of stable isotope reference samples

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Use of light stable isotope ratio measurements has proliferated in the past decade. The need for procuring additional stable isotope reference materials was recognized at an International Atomic Energy Agency (IAEA) consultants' meeting convened in 1976<sup>1</sup>. This group recommended acquisition of two carbonates, two carbon dioxide samples, a biotite, a sulphate, and other reference materials. We report here on the mass-spectrometric analysis of these and other reference samples in a single laboratory to minimize interlaboratory calibration errors. A result of this work is an improved equation for relating the PDB isotope scale (belemnite from the Pee Dee Formation of South Carolina adopted in the 1950s as a reference in palaeotemperature studies<sup>2</sup>) to the V-SMOW (Vienna-Standard Mean Ocean Water) scale<sup>1</sup>.

To provide a means for accurate comparison with analyses by other laboratories we now describe our sample preparation and analysis. Replicate reference water samples were equilibrated at 25.0 °C in a constant shaking bath using 5 ml water and 1.50 mmol carbon dioxide<sup>3</sup>. Replicate carbonates were reacted<sup>4</sup> at 25 °C using 2 ml 100% H<sub>3</sub>PO<sub>4</sub> and 20 mg carbonate. The 100% H<sub>3</sub>PO<sub>4</sub> is prepared by slowly dissolving 2,010 g P<sub>2</sub>O<sub>5</sub> in 4,300 g 85% H<sub>3</sub>PO<sub>4</sub> in a 4-l beaker. Ten mg CrO<sub>3</sub> is added and the solution is covered and heated at 200 °C for 7 h. Three ml H<sub>2</sub>O<sub>2</sub> is added and the solution is heated to 220 °C for an additional 4.5 h. After cooling overnight, the acid is stored in glass bottles with caps containing conical plastic inserts for tight sealing. Because the acid dissolves the black markings on a thermometer, it should be inserted in a glass tube sealed at one end before use. The specific gravity should be between 1.90 and 1.92. If not, more P<sub>2</sub>O<sub>5</sub> or water is added as required and the process is repeated beginning with heating for 7 h. Acid is ready for use after ageing for 1 month.

NBS-21 (National Bureau of Standards no. 21) graphite and NBS-22 oil were prepared by oxidation with CuO at 850 °C in evacuated quartz tubes<sup>5,6</sup>. OGS sulphate was prepared by reaction with powdered graphite at 1,100 °C in a quartz furnace<sup>7–9</sup>.



**Table 1**  $\delta^{18}\text{O}$  values of stable isotope reference samples

| Reference sample | Description     | No. of preparations | Standard deviation ( $1\sigma$ ) | Measured                                      |                                                   | Normalized using $-55.5\%$ for SLAP relative to V-SMOW |                                                   |
|------------------|-----------------|---------------------|----------------------------------|-----------------------------------------------|---------------------------------------------------|--------------------------------------------------------|---------------------------------------------------|
|                  |                 |                     |                                  | $\delta^{18}\text{O}_{\text{PDB-CO}_2}^*$ (‰) | $\delta^{18}\text{O}_{\text{V-SMOW}}^\dagger$ (‰) | $\delta^{18}\text{O}_{\text{PDB-CO}_2}^*$ (‰)          | $\delta^{18}\text{O}_{\text{V-SMOW}}^\dagger$ (‰) |
| V-SMOW           | Water           | 4                   | 0.05                             | -0.29                                         | 0                                                 | -0.26                                                  | 0                                                 |
| NBS-1            | Water           | 4                   | 0.04                             | -8.15                                         | -7.86                                             | -8.16                                                  | -7.91                                             |
| NBS-1A           | Water           | 4                   | 0.03                             | -24.50                                        | -24.21                                            | -24.62                                                 | -24.36                                            |
| SLAP             | Water           | 4                   | 0.07                             | -55.44                                        | -55.16                                            | -55.75                                                 | -55.50                                            |
| NBS-16           | Carbon dioxide  | 3                   | 0.01                             | -35.90                                        | +4.12                                             | -36.09                                                 | +3.89                                             |
| NBS-17           | Carbon dioxide  | 3                   | 0.04                             | -18.62                                        | +22.11                                            | -18.71                                                 | +21.99                                            |
| NBS-18           | Carbonatite     | 3                   | 0.03                             | -22.88                                        | +7.35                                             | -23.00                                                 | +7.20                                             |
| NBS-19           | Marble          | 2                   | 0.02                             | -2.20                                         | +28.67                                            | -2.19                                                  | +28.65                                            |
| NBS-20           | Limestone       | 7                   | 0.02                             | -4.14                                         | +26.67                                            | -4.14                                                  | +26.64                                            |
| NBS-28           | Quartz          | 7                   | 0.06                             | -30.41                                        | +9.84                                             | -30.57                                                 | +9.64                                             |
| NBS-30           | Biotite         | 2                   | 0.02                             | -34.74                                        | +5.32                                             | -34.92                                                 | +5.10                                             |
| OGS              | Barium sulphate | 6                   | 0.06                             | -30.73                                        | +9.51                                             | -30.88                                                 | +9.31                                             |
| TS limestone     | Marble          | 2                   | 0.05                             | -2.25                                         | +28.62                                            | -2.24                                                  | +28.60                                            |

\*  $\delta^{18}\text{O}$  of  $\text{CO}_2$  prepared from reference samples is reported relative to  $\text{CO}_2$  evolved by reaction at  $25^\circ\text{C}$  of  $100\%$   $\text{H}_3\text{PO}_4$  with PDB calcite<sup>4</sup>, assuming  $\delta^{18}\text{O}$  of  $\text{CO}_2$  evolved by reaction at  $25^\circ\text{C}$  of  $100\%$   $\text{H}_3\text{PO}_4$  with NBS-20 is  $-4.14\%$ <sup>2,14</sup>. Water sample data are corrected for the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  used in equilibration procedure.

†  $\delta^{18}\text{O}$  of oxygen in the reference sample is reported relative to V-SMOW water. The  $\text{CO}_2$  water oxygen isotope fractionation factor of 1.04120 recommended by Friedman and O'Neil<sup>14</sup> and the  $\text{CO}_2$ -calcite oxygen isotope acid fractionation factor<sup>13,15</sup> of 1.01025 were used to calculate these data.

Silicates were prepared by reaction with  $\text{BrF}_3$  (ref. 10). NBS-16 and NBS-17 carbon dioxide references<sup>11</sup> required no special preparation.

Mass-spectrometric analysis of each carbon dioxide sample involved duplicate analysis for  $\delta^{45}$ , stepping the accelerator voltage, and triplicate analysis for  $\delta^{46}$  using an automated double-focusing, double-collecting isotope ratio mass spectrometer<sup>12</sup>. The beam monitor on this instrument was used to identify sample contamination by comparing the ratio (total ion beam current)/( $m/e-44$ ) of the sample and standard. Analyses with contamination above 0.25% numbered <1% of the analyses and were discarded. Mass-spectrometric corrections were made after Mook and Grootes<sup>13</sup>. Tail correction and valve mixing corrections are negligible<sup>12</sup>.

Maximum care was taken during all phases of sample preparation and analysis to minimize error. It is well known that Solenhofen limestone, NBS-20, is very fine and will exchange oxygen with atmospheric moisture<sup>14</sup>; therefore, we analysed only newly opened capsules of NBS-20. Additionally, only newly opened ampoules or capsules of other reference materials were analysed. To minimize error due to memory effects, only glass-bore, greased stopcocks were used in water sample equilibration flasks and carbonate reaction vessels.

In accordance with the IAEA consultants' recommendations, we report  $\delta^{18}\text{O}$  values relative to V-SMOW. Our oxygen isotope results, before and after normalization of the data so that  $\delta^{18}\text{O}$  of SLAP (Standard Light Antarctic Precipitation) is  $-55.5\%$  relative to V-SMOW, are shown in Table 1. These results agree very well with those reported by Gonfiantini<sup>1</sup>. Our normalized value of NBS-1 relative to V-SMOW is  $-7.91\%$ ; whereas Gonfiantini reports  $-7.89\%$  as the normalized average of 34 laboratories. No measurable differences were found among the various aliquots of each of the four reference waters in this study. In contrast to results of  $\delta\text{D}$  determinations reported in ref. 14, agreement between analyses of two NBS-1 samples that were sealed in glass ampoules at least 10 yr apart suggest good handling of this reference material over time. Aliquots from three different capsules of NBS-20 showed excellent reproducibility (Table 1). Values reported by several laboratories for  $\delta^{18}\text{O}$  of NBS-28 range from  $+9.2\%$  to  $10.0\%$  (ref. 14). The value herein of  $+9.64\%$  should be decreased by  $0.45\%$  if 1.04073 is used for the  $\text{CO}_2$ - $\text{H}_2\text{O}$  oxygen isotope fractionation factor<sup>16</sup>. This agrees well with the value of  $+9.22\%$  reported by Ito and Clayton<sup>17</sup> using 1.04073.

Oxygen isotope palaeotemperature data are usually reported relative to PDB carbonate<sup>2,14</sup>, the supply of which is now

**Table 2**  $\delta^{13}\text{C}$  values of stable isotope reference samples in ‰ relative to PDB

| Reference sample | Description    | No. of preparations | Standard deviation ( $1\sigma$ ) | $\delta^{13}\text{C}^*$ |
|------------------|----------------|---------------------|----------------------------------|-------------------------|
| NBS-16           | Carbon dioxide | 4                   | 0.03                             | -41.61                  |
| NBS-17           | Carbon dioxide | 8                   | 0.02                             | -4.48                   |
| NBS-18           | Carbonatite    | 6                   | 0.03                             | -5.08                   |
| NBS-19           | Marble         | 7                   | 0.02                             | +1.93                   |
| NBS-20           | Limestone      | 11                  | 0.02                             | -1.06                   |
| NBS-21           | Graphite       | 4                   | 0.03                             | -28.13                  |
| NBS-22           | Oil            | 7                   | 0.03                             | -29.61                  |
| TS limestone     | Marble         | 8                   | 0.02                             | +1.95                   |

\*  $\delta^{13}\text{C}$  relative to PDB assuming  $\delta^{13}\text{C}$  of NBS-20 is  $-1.06\%$  (ref. 2).

exhausted. The PDB scale can be related to the V-SMOW scale through SMOW. However, SMOW is poorly characterized because it has been defined in several different ways over the past three decades, resulting in a multi-valued standard<sup>1,14,18</sup>. Alternatively, NBS-20 analysed by Craig<sup>2</sup> in 1957 as  $-4.14\%$  on the PDB scale has been used for two decades in laboratory calibration and can be used to relate the PDB and V-SMOW scales. Although some workers<sup>19</sup> recently have re-evaluated Craig's results and increased them by 1%, we use the original value of  $-4.14\%$  to maintain consistency with most laboratories<sup>20</sup>. The relationship between the PDB and V-SMOW scales can be determined using: (1) the value of 1.04120 recommended by Friedman and O'Neil<sup>14</sup> for the oxygen isotopic fractionation between carbon dioxide and water at  $25^\circ\text{C}$ ; (2) the value of 1.01025 for the oxygen isotopic fractionation between carbon dioxide evolved from calcite by treatment with  $100\%$   $\text{H}_3\text{PO}_4$  at  $25^\circ\text{C}$  (refs 14, 15); (3) the value of  $-4.14\%$  of  $\text{CO}_2$  evolved by reaction at  $25^\circ\text{C}$  of  $100\%$   $\text{H}_3\text{PO}_4$  with NBS-20 relative to  $\text{CO}_2$  evolved in like manner from PDB calcite<sup>2</sup>; and (4) the value of  $-0.26\%$  for carbon dioxide equilibrated with V-SMOW on the PDB- $\text{CO}_2$  scale reported herein. Thus,

$$\delta^{18}\text{O}_{\text{V-SMOW}} = 1.03091 \delta^{18}\text{O}_{\text{PDB}} + 30.91$$

or

$$\delta^{18}\text{O}_{\text{PDB}} = 0.97002 \delta^{18}\text{O}_{\text{V-SMOW}} - 29.98$$

General adoption of these relationships should help to resolve the confusion in converting  $\delta$ -values from one scale to another by different laboratories.

Table 2 shows the  $\delta^{13}\text{C}$  of reference samples reported relative to PDB by assuming the  $\delta^{13}\text{C}$  of NBS-20 is  $-1.06\%$  (ref. 2). Calibration to the PDB scale was made through NBS-20 because: (1) it was analysed in 1957 by Craig<sup>2</sup> and has been utilized for calibration to PDB in a large number of laboratories since then, and (2) it seems to have excellent reproducibility ( $1\sigma = 0.02\%$ ). The  $\delta^{13}\text{C}$  of NBS-21 of  $-28.13\%$  agrees well with that of  $-28.10\%$  reported by others<sup>4,21,22</sup>. NBS-22 does not seem to contain significant quantities of carbon-bearing volatiles because pumping during sample preparation with or without prior freezing with liquid nitrogen yields the same  $\delta^{13}\text{C}$  of  $-29.61\%$ . In contrast to another report<sup>23</sup>, data in Tables 1 and 2 indicate that NBS-19 and TS Limestone are identical within the error of measurement. It is not unexpected that due to differences in mass spectrometers that determinations of NBS-21 and NBS-22 made in other laboratories may differ by as much as  $0.3\%$  from the values reported herein. Improvement in the coherence among  $\delta^{13}\text{C}$  values reported by different laboratories should be attained by adopting a second reference standard on which to normalize  $\delta^{13}\text{C}$  values<sup>19,20</sup>.

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## Two noble gas components in a Mid-Atlantic Ridge basalt

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**Noble gas patterns in mid-oceanic ridge basalt (MORB) glasses contain important information about the evolution of the Earth<sup>1</sup>. However, such information may be much affected by physical phenomena acting during the transport of deep-seated components to the surface, such as magma vesiculation. Indeed, the development of a gas phase in a silicate melt will induce a preferential partitioning of noble gases in the vesicles, due to their low solubility in silicate melt<sup>2,3</sup> and the existence of such a gas phase is evidenced by the common occurrence of CO<sub>2</sub>-filled vesicles in MORB<sup>4–7</sup>. We have investigated the noble gas distribution within a single vesiculated sample. Our results indicate that two components are present: one, located inside the vesicles, is likely to be mantle-derived; the other, dissolved in the glass, displays evidence of atmospheric contamination.**

The sample, CH31-DR11-NG, is a fresh low-alumina tholeiite which belongs to the same Mid-Atlantic dredge

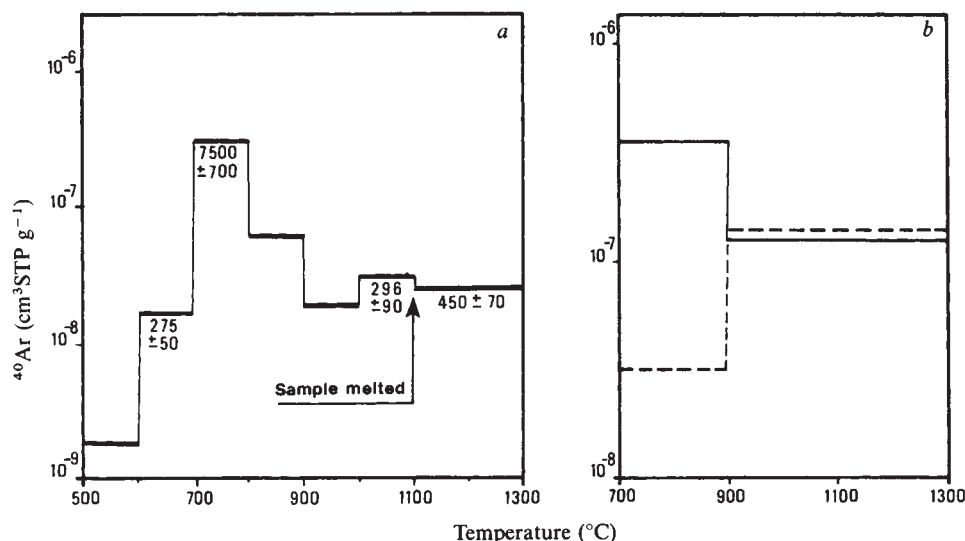
(36° 49' 3N, 33° 15' W) as the so-called "popping rocks"<sup>4,5</sup>. It is characterized by the occurrence of large vesicles (0.2–1.2 mm in diameter), despite an eruption depth of 2,480 m, and the glassy rim selvage is frequently thicker than 1 cm. These observations suggest that the initial volatile content of the magma was high in the sampled area.

Stepwise heating experiments were preferred to vacuum crushing ones to minimize the atmospheric contamination. The glassy part of the sample was separated from a hand specimen and cut into 5–8 mm blocks to preserve the vesicles as far as possible. As is evident in the Ar isotopic analyses (Fig. 1a, Table 1), the sample preparation did not introduce any significant noble gas contamination from the air. A batch of powder (grain size between 0.2 and 0.4 mm) was also prepared by crushing glass. The optimum grain size was chosen from compromise between the two contradicting factors, that is, while extensive crushing of grains is desirable to destroy vesicles, a fine grain size would introduce undesirable atmospheric noble gas contamination. A microscopic observation of the powder did not reveal any of the large vesicles which were observed in the whole glass. A whole block batch was heated by seven temperature steps and, after purification, argon was analysed with a quadrupole mass spectrometer calibrated with known amounts of atmospheric argon. As shown in Fig. 1a, the <sup>40</sup>Ar degassing pattern presents two maxima, one occurring between 700 and 800 °C and the other, probably related to the melting of the sample, registering between 1,000 and 1,100 °C. Two steps were also applied to the powder batch. For the low temperature fraction (700–900 °C), <sup>40</sup>Ar is one order of magnitude more abundant in blocks than in powder, while when both are of a higher temperature fraction, the concentration of argon in both batches is rather similar (Fig. 1b). It is likely that the observed difference resulted from the release of argon trapped in vesicles during decrepitation of the latter. Moreover, the low-temperature excess argon in blocks holds a high <sup>40</sup>Ar/<sup>36</sup>Ar ratio ( $\geq 7,000$ ) and, at the same time, this ratio approximates the atmospheric value of 295.5 at a higher temperature. As argon in the mantle beneath mid-oceanic ridges is characterized by a high <sup>40</sup>Ar/<sup>36</sup>Ar ratio<sup>8</sup>, we infer that CO<sub>2</sub> inclusions have trapped deep-seated argon which did not equilibrate with argon dissolved in the glassy matrix.

To study the behaviour of other noble gases, their elemental abundance as well as the <sup>3</sup>He/<sup>4</sup>He and <sup>40</sup>Ar/<sup>36</sup>Ar ratios were determined using a 6-inch sector type mass spectrometer connected to a metal line. The analytical procedure is described elsewhere<sup>9</sup>. A powder batch was completely melted during a single step (1,500 °C) and three steps (that is, 500, 750, 1,500 °C) were applied to a block batch. The gas release during the 750 °C step, monitored by a Pirani gauge, was characterized by sudden discharges of volatiles, probably resulting from the opening of the vesicles. Another batch of powder was completely melted during a single 1,500 °C step: results are given in Table 1. The argon isotopic ratio varies with the temperature and shows a similar pattern, that is, a high <sup>40</sup>Ar/<sup>36</sup>Ar ratio ( $10,524 \pm 1,047$ ) for the 750 °C step and a comparatively low one for both the 1,500 °C step ( $930 \pm 160$ ) and the powder run ( $385 \pm 14$ ). Conversely, the uniformity of the <sup>3</sup>He/<sup>4</sup>He ratio suggests that an isotopic equilibrium had been attained for helium. This observation is in agreement with other work<sup>10</sup> concerning the He distribution in MORB glasses and may be due to the high mobility of He atoms. These results suggest that noble gases dissolved in the glassy matrix would be released during the powder run as well as during the 1,500 °C block step, while noble gases trapped in the vesicles were probably extracted during the 750 °C block step. Note that a portion of helium and neon may have been extracted from the glass by solid diffusion during the 750 °C step, due to their relatively high diffusion coefficients<sup>10,11</sup>. In contrast, such a diffusion effect is probably limited for argon, and negligible for krypton and xenon. For example, using Ar diffusion data for silicon oxide films<sup>12</sup>, that is,  $D_{\text{Ar}} \approx 10^{-10} \text{ cm}^2 \text{ s}^{-1}$  at 750 °C, we can easily anticipate that the amount of argon released by solid



**Fig. 1** *a*,  $^{40}\text{Ar}$  degassing pattern of sample CH31-DR11-NG (block batch). Mass  $m$  is 1.9174 g. Seven temperature steps each having a plateau temperature lasting 30 min were applied using an induction heater. Purification was performed with a Ti-furnace and a Ti-Zr getter cartridge. Data are corrected for blank ( $6.4 \times 10^{-9} \text{ cm}^3 \text{ STP}$  at  $1,300^\circ\text{C}$  and  $1.5 \times 10^{-9} \text{ cm}^3 \text{ STP}$  at  $1,000^\circ\text{C}$ ). Uncertainty is estimated to be 20%. Numbers shown below the line represent the  $^{40}\text{Ar}/^{36}\text{Ar}$  ratio for the corresponding step. *b*, Comparison of degassing patterns for the powder batch ( $m = 1.5977 \text{ g}$ ; dotted line) and the block batch (solid line). The latter was established using data presented in *a*.



**Table 1** Elemental abundances and isotopic ratios of noble gases

|                                                   | $^{4}\text{He}$<br>( $10^{-7} \text{ cm}^3 \text{ STP g}^{-1}$ )  | $^3\text{He}/^4\text{He}$       | $^{20}\text{Ne}$<br>( $10^{-10} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^{36}\text{Ar}$<br>( $10^{-10} \text{ cm}^3 \text{ STP g}^{-1}$ )  |
|---------------------------------------------------|-------------------------------------------------------------------|---------------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------|
| Powder batch<br>$m = 0.5766 \text{ g}$ (1,500 °C) | 4.62 (0.04)                                                       | $1.01 \times 10^{-5}$           | 2.1 (1.0)                                                          | 1.80 (0.40)                                                         |
| Block batch, $m = 0.5455 \text{ g}$               |                                                                   |                                 |                                                                    |                                                                     |
| 1st step (500 °C)                                 | 0.42 (0.04)                                                       | —                               | ND (0.6)                                                           | ND (0.24)                                                           |
| 2nd step (750 °C)                                 | 68.13 (0.04)                                                      | $1.11 \times 10^{-5}$           | 7.3 (0.7)                                                          | 1.03 (0.26)                                                         |
| 3rd step (1,500 °C)                               | 1.75 (0.04)                                                       | $1.12 \times 10^{-5}$           | 0.6 (0.9)                                                          | 0.32 (0.31)                                                         |
| Total                                             | 70.30                                                             | $1.11 \times 10^{-5}$           | 7.9                                                                | 1.35                                                                |
|                                                   | $^{40}\text{Ar}$<br>( $10^{-7} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^{40}\text{Ar}/^{36}\text{Ar}$ | $^{84}\text{Kr}$<br>( $10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^{132}\text{Xe}$<br>( $10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ ) |
| Powder batch<br>$m = 0.5766 \text{ g}$ (1,500 °C) | 0.69 (0.13)                                                       | $385 \pm 14$                    | 8.31 (2.52)                                                        | 3.02 (0.18)                                                         |
| Block batch, $m = 0.5455 \text{ g}$               |                                                                   |                                 |                                                                    |                                                                     |
| 1st step (500 °C)                                 | 0.04 (0.10)                                                       | —                               | 0.75 (1.20)                                                        | 0.15 (0.06)                                                         |
| 2nd step (750 °C)                                 | 10.84 (0.12)                                                      | $10,524 \pm 1,047$              | 3.62 (1.35)                                                        | 0.30 (0.07)                                                         |
| 3rd step (1,500 °C)                               | 0.30 (0.14)                                                       | $930 \pm 160$                   | 0.88 (1.73)                                                        | 0.34 (0.10)                                                         |
|                                                   | 11.18                                                             | 8,282                           | 5.25                                                               | 0.79                                                                |

Each step took 30 min at constant temperature. All data are corrected for blanks whose corresponding values are given in parentheses. ND, Not detected. The line and the 6-inch sector type mass spectrometer were calibrated with five atmospheric noble gas runs. The accuracy on elemental abundance is typically 10%. Due to the low resolution power of the mass spectrometer (150) for these experiments, the  $^3\text{He}$  peak was not completely separated from the HD peak. Thus, precision on the  $^3\text{He}/^4\text{He}$  ratio should be  $\sim 10\%$ .

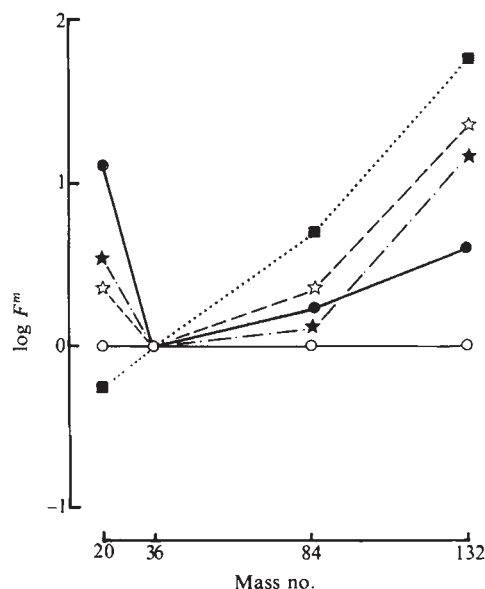
diffusion from a glass cube of 5-mm side during 30 arc min will be lower than 1%.

The noble gas elemental fractionation pattern of the  $750^\circ\text{C}$  step (after normalization to the atmospheric pattern, see Fig. 2) is different from that of the powder run and from that of the  $1,500^\circ\text{C}$  block step, the latter two being enriched in xenon. As the assimilation of atmospheric noble gases, through adsorption, sedimentary or seawater contribution, enhances heavy noble gases with respect to lighter ones<sup>13,14</sup>, the powder run and  $1,500^\circ\text{C}$  block step patterns probably resulted from atmospheric contamination. In the case of powder, this contamination is possibly due to surface adsorption during the preparation of the sample. This may explain the relatively large noble gas abundance observed for this batch (Table 1). However, such a phenomenon cannot be claimed in the case of the  $1,500^\circ\text{C}$  block step, as the surface of the blocks in contact with the atmosphere is much smaller than that of the powder grains. Moreover, these blocks were degassed during previous temperature steps. Thus the glassy matrix was probably already contaminated before deep-sea sampling. This inference is

strongly supported by the low  $^{40}\text{Ar}/^{36}\text{Ar}$  ratio (close to the atmospheric value) observed in the glass.

The observed disequilibrium between vesicles and glass is possibly due to a greater contamination effect in the case of the melt, this being apparently more depleted in mantle component than the vesicles. However, vesicles were possibly added after contamination of the melt. Such a mixing is obviously impossible after eruption, that is, quenching of the lava, and must have occurred in the magma reservoir, as rising  $\text{CO}_2$  bubbles encountered in a portion of the melt contaminated by atmospheric components, located for example in the upper part of the magma chamber or in the output dykes. The argon isotopic disequilibrium implies that the duration of contact between  $\text{CO}_2$  bubbles and melt must have been rather limited.

Although the above discussion is still conjectural, our results indicate that a simple exsolution model cannot account for the argon isotopic disequilibrium. This implies that one should pay special attention when dealing with basalt glass and interpreting such data. In particular, crushing of samples before analysis may induce a significant loss of mantle-derived noble gases.



**Fig. 2** Fractionation patterns of noble gases with respect to atmospheric one for sample CH31-DR11-NG. Factor  $F^m$  is defined as follows:

$$F^m = ({}^mX/{}^{36}\text{Ar})_{\text{sample}} / ({}^mX/{}^{36}\text{Ar})_{\text{air}}$$

where X represents a non-radiogenic noble gas isotope of mass number  $m$ . The CH31-DR11-NG sample patterns are compared with that of Fig Tree Shale<sup>14</sup>. The latter one shows the effect of preferential adsorption of atmospheric noble gases. ○, Atmosphere; ☆, powder run; ●, 750°C block step; ★, 1,500°C block step; ■, Fig Tree Shale.

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## A new model for the Giant's Causeway

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O'Reilly's (1879) data for the columnar structure of the Giant's Causeway exhibit a strong correlation of neighbouring sides and angles which has not been previously noted. It illustrates the nature of the problem posed by the Causeway, in that the crack network responsible for the structure, although irregular, is remarkably homogeneous. We argue here that existing models are inadequate and propose a new one, in which the dynamics of vertical crack propagation have an essential role.

**Table 1** Correlation of lengths and angles (855 data points)

|                                                         | Mean,<br>linear | s.d.     | Correlation<br>coefficient |
|---------------------------------------------------------|-----------------|----------|----------------------------|
| Length, $L$ (cm)                                        | 27.2            | 9.1      |                            |
| Angle, $\theta$ (deg)                                   | 120.4           | 13.8     | 0.75                       |
| Linear regression of angle on length, $\theta = mL + C$ |                 |          |                            |
|                                                         |                 | Estimate | s.e.                       |
| Regression coefficient, $m$                             |                 | 1.14     | 0.03                       |
| Intercept, $c$                                          |                 | 89.5     | 1.03                       |

The Giant's Causeway has been a subject of speculation for three centuries, and although we may discard most of the more primitive theories some textbooks still quote the early work of Mallet<sup>1</sup>, which purported to demonstrate that a hexagonal crack network is ideal. The observed structure is not hexagonal, but is rather an irregular network, which seems to be broadly similar to that found in other locations<sup>2</sup>. Moreover, Mallet's calculations are, if examined in detail, difficult to analyse or accept, even if his approach is based on the now accepted mechanism of crack formation due to cooling on contraction.

Smalley's more recent model<sup>2</sup> uses a purely geometrical construction (Voronoi polygons of points distributed in a random manner, with some conditions.) It lacks a convincing physical basis and achieves, according to Getis and Boots<sup>3</sup>, only a vague resemblance to the observed structure. The dominant theme of most modern qualitative descriptions which involve an explicit process has been horizontal crack propagation and bifurcation<sup>4,5</sup>. This bears no clear relation to either Mallet's or Smalley's calculations and is not amenable to much theoretical development even today<sup>5</sup>.

The crack network displays a remarkable homogeneity, balance and consistency throughout, which seems incompatible with formation by the horizontal propagation and bifurcation of cracks<sup>5</sup>. One would expect that the more or less accidental confluence of cracks would generate a lot of 'mistakes' even if they emanated from a single centre. The resulting structure would be inhomogeneous, due to the mismatch of propagating cracks in some areas.

This subject has always suffered from a reliance on vague descriptions of the Causeway (as above), rather than any precise diagram of it, or any statistical measure of its structure beyond the distribution  $f(n)$  of  $n$ -sided columns. It has been generally overlooked that O'Reilly made a detailed survey of an area comprising over 200 columns in the 1870s and that it was published in a very complete form<sup>6</sup> that should be the basis for any attempt to develop criteria for a satisfactory model, as well as a useful reference when considering such matters as the frequency of occurrence of fourfold vertices<sup>7</sup>. In particular, inspection of O'Reilly's map suggests that there is throughout the structure a close correlation of the angles of vertices with the lengths of neighbouring sides ( $L$  and  $\theta$  in Fig. 1). In what follows we show that the data support such a relationship.

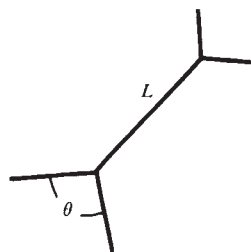
A linear regression analysis was carried out on 855 pairs of angles and sides taken from O'Reilly's data. The results are set out in Table 1 and illustrated in Fig. 2. The data reveal that there is indeed a significant correlation between  $L$  and  $\theta$ . To within the standard error of the regression analysis, this may be expressed as

$$\theta = 90 + 30L/L_{\text{mean}} \text{ (deg)}$$

where  $L_{\text{mean}}$  is the mean side length. Note that fourfold vertices conform neatly to such a relation if we consider them to correspond to  $L = 0$  in which case the appropriate average angle is  $360/4 = 90^\circ$ .

Such a relation is by no means a universal feature of disordered cellular structures in two dimensions. It is easy to invoke counter-examples—for example, one can construct networks in which lengths fluctuate but all angles are fixed at  $120^\circ$ , or in which angles fluctuate but lengths do not.





**Fig. 1** Definition of neighbouring sides  $L$  and angles  $\theta$  used in the analysis.

With the absence of any detailed model in the literature, it is difficult to test the propagation-bifurcation model quantitatively in terms of this relation, but it seems unlikely that it could produce a network which obeys such a relation so well. One would expect values of  $L$  and  $\theta$  to be concentrated around fixed ideal values with occasional and essentially random values due to accidental features.

How then could such a disordered but strongly correlated structure arise? We propose that it was formed initially in a more irregular form at an interface, by horizontal crack propagation and bifurcation<sup>5</sup>, as in the existing model, but evolved significantly during the subsequent vertical propagation of the existing cracks into the interior of the basalt. The cracks would tend towards a dynamically stable arrangement which would then propagate unchanged. The precise local conditions for such dynamical stability are difficult to specify, although in principle they might be investigated, but it seems likely that they do not demand a regular (periodic) structure. Rather they would impose local correlations of crack configurations, such as those that we have noted here. Note that no inhomogeneity of the basalt is required to account for the irregularity of the network. Moreover, given a sufficiently disordered initial crack system, the stable crack network produced might be expected to have statistical properties independent of the initial state—hence the similarity of basalt columnar structures in different locations<sup>2</sup>.

The process of evolution of the structure towards a stable arrangement which we have suggested resembles that by which

a glass forms as a homogeneous random network on cooling a liquid, except that we are dealing here with the more difficult case of local dynamical stability of the crack configuration.

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## Pollutant transfer in upland regions by occult precipitation

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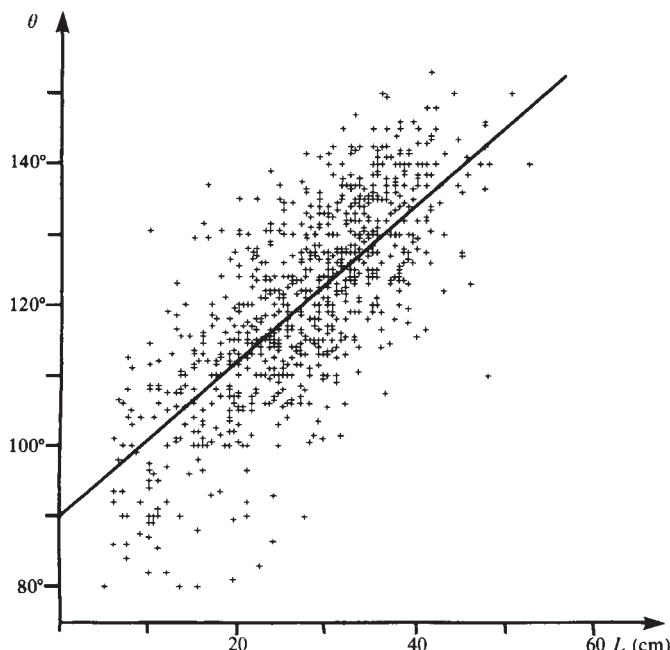
**Pathways for the transfer of pollutants from the atmosphere to vegetation by gaseous absorption (dry deposition) and in rain (wet deposition) have been studied extensively. Here we quantify deposition by a third pathway, that which occurs when vegetation exposed in wind-driven cloud intercepts water drops which are not collected efficiently in standard rain gauges<sup>1</sup>. From measurements of the chemical content and rate of this 'occult precipitation'<sup>2</sup> at Great Dun Fell, Cumbria in the United Kingdom, we make preliminary estimates of the deposition of several elements. We tentatively conclude that the inclusion of the occult deposition pathway in assessing annual chemical deposition in areas prone to low cloud could increase wet deposition estimates by up to 20% from values recorded in conventional rainfall chemistry gauges alone.**

The hydrological significance of occult precipitation, which includes water deposited through the interception of fog, mist and cloud, has been recognized for many years<sup>3</sup> though rarely quantified. In mountain ranges or coastal areas where rain is infrequent but where orographic cloud cover or advective fog is common, occult precipitation may be a significant source of water<sup>1,3</sup>.

Cloud and fog drops may contain a wide range of elements, often at concentrations considerably greater than those for rainfall collected in the same area<sup>4–7</sup>. This is most likely a consequence of the drops being sampled or impacted at a relatively early stage in their development. Pollutant aerosols or particles act as condensation nuclei, thus chemical concentrations in cloud or mist drops are relatively higher than in rain drops when growth and dilution have taken place by condensation mechanisms.

The measurements reported here were made at Great Dun Fell (850 m elevation) in Cumbria, a site which frequently experiences cloud cover. Fluxes of liquid water to the surface were measured on 17 June 1982 using a micrometeorological technique based on the aerodynamic method<sup>8</sup> and reported fully elsewhere<sup>9</sup>. At the experimental site, near to the fell top, fetch was across several miles of rolling terrain with a moderate slope. The vegetation was sparse consisting mainly of short grass, roughened by areas of exposed peat, surface rocks and moorland plants<sup>10</sup>.

The mean cloud drop flux to the surface vegetation was  $20 \text{ mg m}^{-2} \text{ s}^{-1}$ . Simultaneously with the flux measurements, samples of cloud water were collected using a fog gauge based on the 'harp' collector described by May<sup>11</sup>. Cloud water was



**Fig. 2** Data used in the regression analysis. The line shown is  $\theta = 90 + 30L/L_{\text{mean}}$ , which is very close to the relation obtained from the regression analysis.

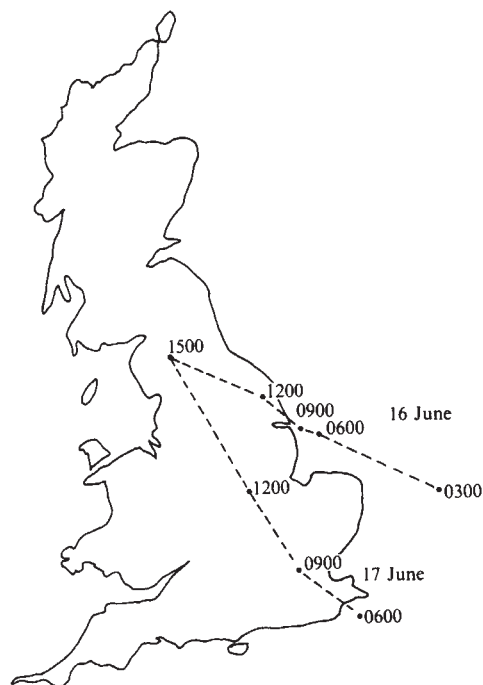


Fig. 1 Trajectories of air arriving at Great Dun Fell on 16 and 17 June 1982.

also collected on 16 June 1982, and samples were stored at 1 °C in the laboratory until analysed. Sulphate determinations were made using thoronol reagent in a continuous flow autoanalyser system after removal of interfering cations. Nitrate was determined using an ion specific electrode. Remaining analyses were performed using atomic absorption spectroscopy.

Trajectory analyses of the air arriving at the fell during the sampling periods used surface UK charts giving 3-hourly readings of pressure and wind direction. Allowance was made for differences between surface and geostrophic wind directions.

Table 1 shows results of the chemical analyses: concentrations are high in comparison with typical averages for rainfall for the area<sup>12,13</sup>. The hydrogen ion content of samples taken on both days corresponds to the most severe acid-precipitation reported for Scandinavia<sup>14</sup>, North America<sup>15</sup> and Scotland<sup>16</sup>.

Hourly deposition rates in Table 1 are based on the flux measurements made on 17 June 1982. The data show that deposition in occult precipitation of nitrate, recently identified as an important constituent of acid precipitation in Scotland<sup>16</sup>, is of comparable magnitude to sulphate deposition.

Figure 1 shows trajectory analyses which indicate possible source regions of the deposited elements. On 17 June 1982 the air moved across major pollution sources in the south-east and midlands of Britain. Air on 16 June 1982 had a shorter land track, passing over industrial areas of the east coast. The higher concentrations evident for 16 June may be partly explained by the slower rate of air movement, giving more time for pollutant loading to occur. Although back projection of the tracks for more than 12 h is imprecise it appears that the air on 16 June may have crossed other industrialized regions of FRG and the Netherlands.

The significance of occult deposition on an annual basis depends on several factors including drop deposition rates, chemical concentrations and frequency or duration of the periods of low cloud. From the present study we have estimates for the first two factors; the frequency and duration of low cloud in the Great Dun Fell region depends critically on height.

Meteorological recordings made at Moorhouse (5 km from Great Dun Fell, elevation 560 m), for the years 1973 to 1977 indicate that on average there were 45 days per year on which fog or low cloud was recorded. At the field station on the top of the fell, cloud occurs on parts of 250 days per year<sup>17</sup>. For the purposes of estimation, we therefore assume 150 days per year of fog and low cloud cover and an average persistence of 4 h per day. Thus the total number of 'cloud hours' per year is taken as 600.

Data from the Carlisle meteorological station for 1979 indicate that the wind blew from a sector from east to south for 34% of the time. Consequently the number of cloud hours when air would have moved over major sources of pollution would be about 200 per year. Assuming that chemical deposition is negligible from other wind directions, annual deposition rates for each element may be estimated from Table 1 and are summarized in Table 2. These rough calculations are probably underestimates because of the conservative assumptions listed previously, but clearly it is necessary to make measurements over a longer period to reduce the uncertainties involved in this extrapolation.

Table 2 also compares the estimated deposition in occult precipitation with annual deposition in rainfall to the area. Annual deposition through occult precipitation may be a substantial fraction of the total attributed to wet deposition. Wet deposition measurements made in conventional rain gauges would underestimate total sulphur deposition by about 15% at this site. Similarly, underestimates for nitrogen and lead deposition would occur (Table 2).

It is evident that occult deposition is potentially an important pathway for some pollutants in areas exposed to wind-driven cloud. As conventional gauging does not account for this extra input, current measurements of acidic rainfall in areas prone to occult precipitation may well be underestimates. These

Table 1 Summary of cloud water chemical analyses and calculated deposition rates

|                                       | Date         | SO <sub>4</sub> | NO <sub>3</sub> | Pb   | Elements analysed |      |       |      |                | pH   |
|---------------------------------------|--------------|-----------------|-----------------|------|-------------------|------|-------|------|----------------|------|
|                                       |              |                 |                 |      | Mg                | Fe   | Cu    | Cd   | H <sup>+</sup> |      |
| Cloud water content                   | 16 June 1982 | 123             | 149             | 1.05 | 6.46              | 0.44 | 0.49  | 0.05 | 310*           | 3.50 |
| (µg ml <sup>-1</sup> )                | 17 June 1982 | 55              | 41              | 0.17 | 0.36              | 0.23 | 0.10  | —    | 450*           | 3.35 |
| Deposition rates                      | 17 June 1982 | 4               | 3               | 0.01 | 0.03              | 0.02 | 0.007 | —    | 0.03           |      |
| (mg m <sup>-2</sup> h <sup>-1</sup> ) |              |                 |                 |      |                   |      |       |      |                |      |

\*  $\mu\text{equiv. l}^{-1}$ .

Table 2 Estimated annual inputs of elements to Moorhouse from cloud water interception and rainfall ( $\text{kg ha}^{-1} \text{yr}^{-1}$ )

|             | S   | N     | Pb    | Mg   | Fe   | Cu   | Cd     | H <sup>+</sup> |
|-------------|-----|-------|-------|------|------|------|--------|----------------|
| Cloud water | 2.7 | 1.4   | 0.02  | 0.06 | 0.04 | 0.01 | —      | 0.07           |
| Rainfall    | 15* | 14.0† | 0.09‡ | 3.2§ | 1.05 | 0.14 | <0.04‡ | 0.50–1.0       |

The values shown are based on measurements made on 17 June 1982.

\* Ref. 18; † ref. 19; ‡ ref. 13; § ref. 16.



underestimates would be most significant in forested regions because wind-driven drops are captured more efficiently by trees than by short vegetation.

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## A reinterpretation of the amphisbaenian orbitosphenoid

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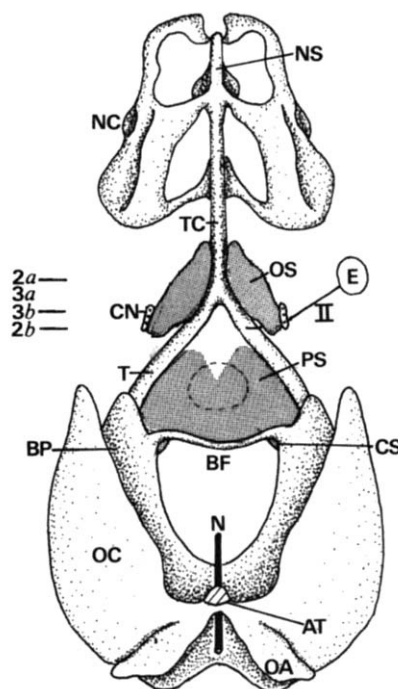
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The Amphisbaenia is a successful group of some 140 species of strange, specialized burrowing reptiles with reduced eyes and limbs, found mainly in parts of Africa and America. Their systematic position has been much debated<sup>1</sup>. They show many unique features, including a large orbitosphenoid bone which has previously been regarded as a cartilage ossification<sup>2</sup>, like that of vertebrates in general<sup>3</sup>. However, appropriate embryonic material available for the first time shows that it is a membrane bone. This remarkable condition tends to emphasize the distinct status of the Amphisbaenia within the Squamata, as a sister-group equivalent to the lizards or the snakes<sup>1,4,5</sup>.

In most lizards the orbital region of the chondrocranium consists of the interorbital septum which is partly derived from the fused trabeculae (trabecula communis): the planum suprasetale, a trough-like structure which supports the fore-brain, and more posteriorly, a scaffolding of bars which like the planum belong to the orbital cartilage system. Some calcification of the orbital cartilages may occur in maturity, and parts of them may ossify to form small paired orbitosphenoids; in general, however, this region of the chondrocranium remains cartilaginous throughout life<sup>2</sup>.

The orbitosphenoid (fused orbitosphenoids<sup>6</sup>) of adult amphisbaenians, however, is a substantial plate of bone which forms the floor of the anterior part of the cranial cavity. It lies dorsal to the rod-like trabecula communis (there is no proper interorbital septum) and to the palatines, ventral to the descending flanges of the frontals and parietals, and mainly anterior to the parasphenoid. In some forms, at least, it is pierced by foramina for the tiny optic nerves.

Comparison with lizards would suggest that this orbitosphenoid is a replacement ossification of the planum suprasetale

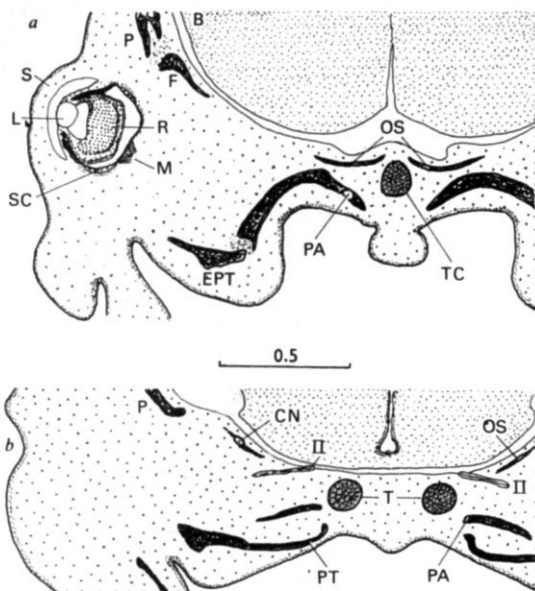


**Fig. 1** *Leposternon microcephalum*. 5 mm head-length embryo. Simplified reconstruction of chondrocranium in dorsal view, also showing orbitosphenoid and membranous (parasphenoid) component of parabasisphenoid bone. Approximate levels of sections in Figs 2 and 3 shown on the left. AT, Anterior process of tectum (cut); BF, basicranial fenestra; BP, basal plate; CN, cartilaginous nodule associated with orbitosphenoid; CS, crista sellaris; E, eye (shown on right only); N, notochord (cut posteriorly); NC, nasal capsule; NS, nasal septum; OA, occipital arch; OC, otic capsule; OS, orbitosphenoid; PS, parasphenoid (outline of pituitary region in broken lines); T, trabecula; TC, trabecula communis; II, optic nerve.

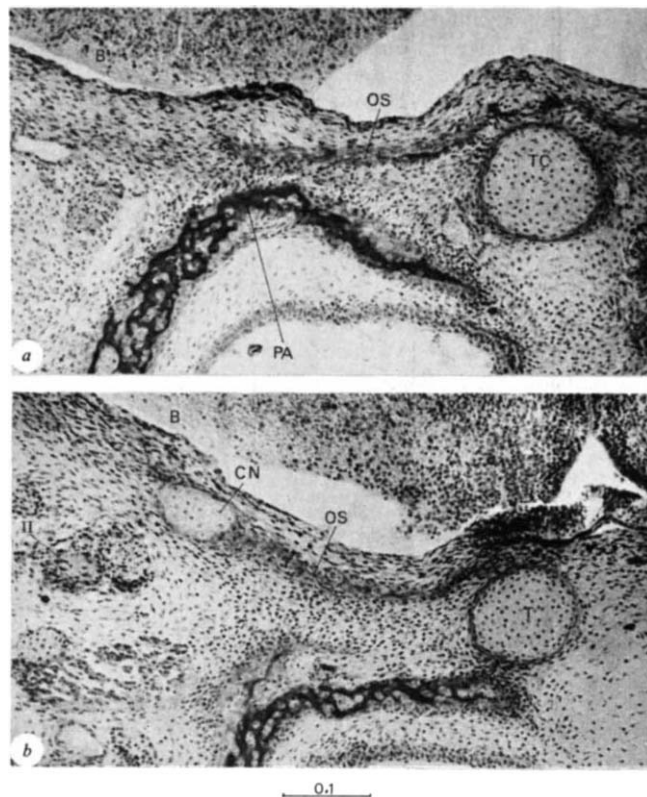
and adjacent parts of the orbital cartilage system<sup>2</sup>, and hence a relatively minor modification of the basic saurian pattern. However, all considerations of this region of the amphisbaenian chondrocranium have been based on postnatal material (and on one apparently very late embryo<sup>7</sup>). We have been able to obtain embryos of three stages of the South American species *Leposternon microcephalum*, and these strongly suggest that the nature of the orbitosphenoid has been misinterpreted.

An embryo of 5 mm head-length provides the most critical stage for interpretation (Fig. 1). The floor of the orbital region of the cranial cavity is formed by two thin sheets of bone, one on either side of the midline. Their medial edges overlap the trabecula communis (Figs 2a, 3a) and the anterior parts of the paired trabeculae (Fig. 3b). The optic nerves pass ventral, and then medial to these bones to reach the brain (Figs 1, 2b). The position of these bones relative to the surrounding cranial elements and to the optic nerves leaves little doubt about their identities. Later fusion in the midline would give rise to the unpaired orbitosphenoid of the adult, and such fusion has partly occurred in an older, 6.5 mm head-length embryo. Owing to this fusion the optic nerves come to pass through discrete foramina in the definitive bone.

The histological appearance of the orbitosphenoids in the 5 mm embryo, stained with Masson's trichrome, strongly suggests that they are essentially membrane bones. They appear as thin plates of ossification within mesenchyme (Fig. 3a) and (except at one site; see below) do not resemble perichondral or endochondral ossifications replacing preexisting orbital cartilages. Indeed, these cartilages appear to be virtually absent in amphisbaenians, both in our embryonic material and in adults; as in snakes, the orbital region of the chondrocranium is represented entirely or almost entirely by trabecular derivatives.



**Fig. 2** *Leposternon*, 5 mm head-length embryo. *a*, Transverse section through left side of head at level of eye. *b*, Section at more posterior level showing optic nerves approaching brain. B, Brain; N, posterior extremity of cartilaginous nodule, associated with orbitosphenoid; EPT, ectopterygoid; F, frontal; L, lens; M, eye muscles; OS, orbitosphenoid; P, parietal; PA, palatine; PT, pterygoid; R, retina; S, spectacle; SC, scleral cartilage; T, trabecula; TC, trabecula communis; II, optic nerve. Other nerves and muscles not shown. Scale in mm.



**Fig. 3** *Leposternon*, 5 mm head-length embryo. *a*, Transverse section through left orbitosphenoid at similar level to Fig. 2*a*. *b*, Section between levels of Fig. 2*a* and *b* showing orbitosphenoid and associated cartilaginous nodule, and the left trabecula. Scale in mm. Lettering as in Fig. 2.

However, in the 5 mm head-length embryo there is a pair of small cartilaginous nodules, one on the posterolateral aspect of each orbitosphenoid (Figs 1, 3*b*). Each nodule seems to be undergoing perichondral ossification in continuity with the much more extensive ossification of the membrane bone. The nodules cannot make more than a small contribution to the definitive orbitosphenoid; it seems likely that they represent vestiges of the orbital cartilages of *Sphenodon* or lizards, such as are sometimes seen in the embryos of certain snakes<sup>2</sup>.

It would therefore appear that the orbitosphenoid of *Leposternon*, and probably of other amphisbaenians (since the adult bone shows relatively minor variation) is largely or entirely a true membrane bone in the illuminating but as yet somewhat unfamiliar sense of Patterson<sup>8</sup>. Such bones ossify in membrane deep in connective tissue, away from the skin. Despite their ontogeny they may be phylogenetically homologous with cartilage bones in other forms. Patterson regards them as distinct from elements such as the frontals and parietals, which have traditionally been called membrane bones<sup>3</sup>, but for which the term dermal bone is more appropriate because they are phylogenetically associated with the skin. He writes that while true membrane bones are not uncommon in bony fishes, their occurrence in tetrapods is doubtful and still a subject for investigation. The orbitosphenoid of *Leposternon* seems to conform with Patterson's definition of a membrane bone. Possibly a new term might be used, but we have retained the name 'orbitosphenoid' here.

We conclude that the amphisbaenian orbitosphenoid is of paired origin<sup>6</sup>, but, contrary to some previous accounts<sup>2</sup>, is usually, if not always, unpaired in the adult. It is important in reinforcing the floor of the anterior braincase and hence in giving the skull the box-like rigidity which, in amphisbaenians at least, is a feature of burrowing adaptation. A parallel enclosure of the cranial cavity is found in snakes, which may also be of burrowing origin<sup>9</sup>, but here the bony floor of the cranium between the eyes is formed entirely by the descending flanges of the frontals and parietals, and mid-ventrally by the

rostrum of the parasphenoid; there are no orbitosphenoid bones.

Among many lizards examined we have observed comparable ossification of the floor of the anterior braincase only in microteiids of the genus *Bachia*. Serial sections of adult *Bachia* (formerly *Ophiognomon*<sup>10</sup>) *trisanale* show this bone attached to the posterior edge of the cartilaginous planum supraseptale on each side; possibly it arises as an intramembranous extension from the planum. To some extent the bone resembles the paired orbitosphenoid of embryonic *Leposternon*. This finding is of interest since a relationship between microteiids and amphisbaenians has been postulated<sup>11</sup>. In other respects, however, the cranial anatomy of microteiids differs considerably from the amphisbaenian condition. The orbitosphenoids of *Bachia* are at present best interpreted as parallel developments, though their discovery supports the view that the morphology of the microteiids and of other fossorial squamates deserves further attention<sup>1</sup>.

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## Two methods of catecholamine depletion in kitten visual cortex yield different effects on plasticity

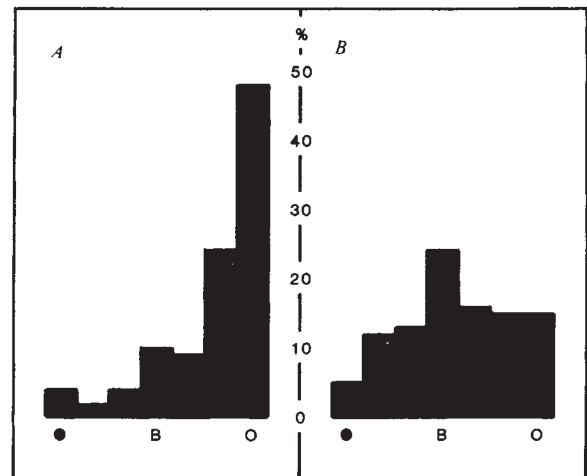
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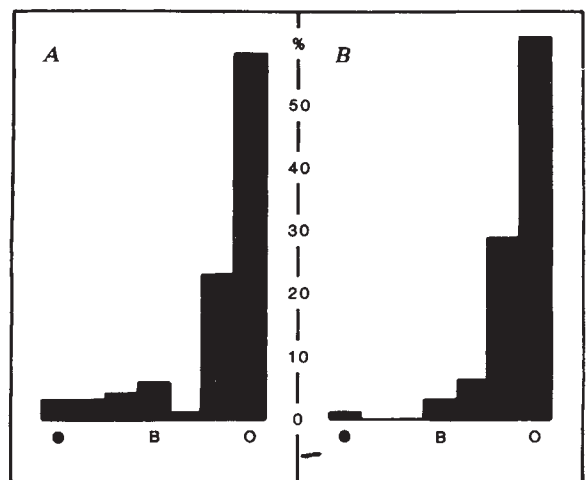
As first clearly demonstrated by the experiments of Wiesel and Hubel<sup>1</sup>, the developing visual cortex is exquisitely sensitive to sensory deprivation. Temporary closure of one eye of a kitten during a critical period that extends from 3 weeks to 3 months of age results in a dramatic cortical reorganization such that most neurones, originally binocularly driven, are dominated exclusively by the open eye. Recently, attention has been directed to chemical factors which may influence the degree of plasticity during the critical period. The work of Kasamatsu and Pettigrew<sup>2,6</sup> suggests that cortical catecholamines, especially noradrenaline (NA), are essential for the normal plastic response to visual deprivation. In an effort to clarify the role of NA in visual cortical plasticity, we have monocularly deprived kittens whose cortex had been depleted of catecholamines by the neurotoxin 6-hydroxydopamine (6-OHDA)<sup>7-9</sup>. We used two strategies to deplete cortical NA: the first, pioneered by Kasamatsu *et al.*<sup>5</sup>, utilized osmotic minipumps to deliver 6-OHDA to visual cortex; the second involved systemic neonatal injections of 6-OHDA, a technique which has proved effective in rodents<sup>10-12</sup>. We found, using high-pressure liquid chromatography (HPLC), that both techniques produced a substantial reduction in the level of cortical NA. However, single unit recording in area 17 revealed that the plastic response to monocular deprivation (MD) was only diminished in the kittens depleted by minipump.

In the first group of kittens ( $N=10$ ) we followed the 6-OHDA delivery procedures of Kasamatsu *et al.*<sup>5,6</sup>. Animals normally reared to 4-8 weeks of age were fitted with two osmotic minipumps (Alza) connected to cannulae inserted in each hemisphere, 0.5-2.5 mm below the cortical surface, near the areae centrales representations in area 17. One pump contained 4 mM 6-OHDA in vehicle (0.4% ascorbate in saline) and the other contained vehicle alone. Seven of these kittens were used for physiological analysis. In this group of kittens, one hemisphere served as control for the other. Each animal in a second group of kittens ( $N=7$ ) was given two intraperitoneal injections of 6-OHDA within 48 hours after birth (doses appear in Table 2). Five animals in this group were used for neurophysiological analysis. Littermates received neonatal injections of vehicle and served as a control group.

Minipump-fitted kittens received 7 days of monocular deprivation concurrently with their drug delivery, starting between 4 and 8 weeks of age (Table 1). In the neonatal series, four 6-OHDA treated kittens and four littermate controls received 10 days of monocular deprivation between 4 and 6 weeks of age. In addition, one 6-OHDA treated kitten and its littermate control were deprived for over 1 month (Table 2). The physiological recording procedure<sup>13,14</sup> was identical for all kittens studied. Receptive fields of visually responsive cells in area 17 were plotted and ocular dominance discriminations were made according to the criteria of Hubel and Wiesel<sup>15</sup>. In the minipump kittens, recording sites were 0.5-2 mm anterior to the cannulae sites. Our recording techniques were similar to those of Kasamatsu and Pettigrew except that we recorded from both hemispheres simultaneously (two electrodes mounted in the same microdrive advance) and we recorded blind, without knowledge of the animal's drug history. The codes were broken after single unit recording.



**Fig. 1** Composite ocular dominance histograms of six kittens in the minipump 6-OHDA series. 6-OHDA was delivered by osmotic minipump to visual cortex of one hemisphere for 1 week while the animal was monocularly deprived. The opposite hemisphere received vehicle solution. **A**, The percentage of cells found in each of the seven ocular dominance groups in the control hemispheres. The open circle is under the monocular open eye group, the filled circle is under the monocular closed eye group, and B labels the strictly binocular group (group 4). As expected, a substantial shift of neurones to the open eye is observed. **B**, The data obtained from the drug-treated hemispheres. In contrast to the controls, most cells in these hemispheres were binocularly activated and the ocular dominance shift was slight.



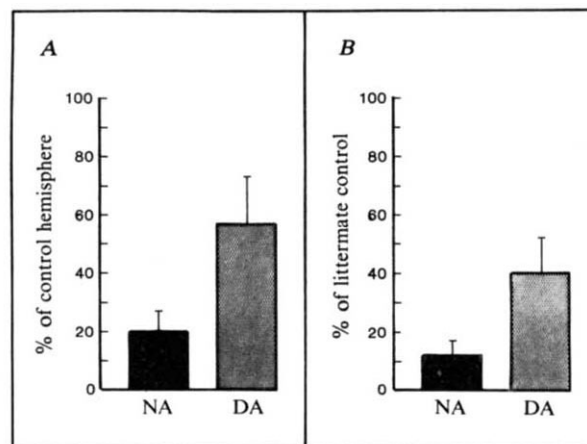
**Fig. 2** Ocular dominance histograms from the neonatal 6-OHDA series. In this series, kittens were injected intraperitoneally as neonates with 6-OHDA. Littermates served as vehicle injected controls. **A**, The composite histogram from the control kittens. These animals displayed the expected shift to the open eye. **B**, Data obtained from drug-treated kittens. Unlike the 6-OHDA-treated hemispheres of the minipump series, these kittens displayed a complete ocular dominance shift, virtually indistinguishable from control. All conventions are as in Fig. 1.

Following each recording session, samples of cortical tissue from near the electrode sites were removed and frozen overnight at  $-30^{\circ}\text{C}$ . In the minipump kittens each sample was a 5 mm length of the postlateral gyrus measured posterior to the site of drug infusion (mean wet weight 102 mg). In the systemically treated neonates, the samples were larger (mean wet weight 1.46 g) and included all of cortical areas 17 and 18. Tissue catecholamines were purified by a batch aluminium oxide chromatography technique based on a column procedure described by Anton and Sayre<sup>16</sup>. Dihydroxybenzylamine (DBA) was added as a standard to monitor recovery. Aliquots

of alumina purified samples were applied to an HPLC column (Bioanalytical Systems) and the separated catechols (approximate retention times: NA, 7 min; DBA, 14 min; dopamine (DA), 24 min) were measured by an electrochemical detector<sup>17</sup>.

The ocular dominance results for the six minipump-fitted kittens that were successfully infused with 6-OHDA are shown in Table 1 and Fig. 1. Neurones from the control hemispheres overwhelmingly preferred stimulation through the open eye. However, in the 6-OHDA treated hemispheres we found many more binocular neurones and the ocular dominance shift was greatly attenuated. The biochemical analysis confirmed the effectiveness of the drug treatment: 6-OHDA treated tissue contained an average of only 21% the NA of controls (Table 1 and Fig. 3A). We conclude that the minipump delivery of 6-OHDA, introduced by Kasamatsu and Pettigrew, indeed depletes cortical NA and diminishes the plastic response to monocular deprivation.

Figure 2 illustrates pooled ocular dominance results for kittens injected with 6-OHDA at birth compared with vehicle-injected littermates. Data from individual kittens are shown in Table 2. Again, the control kittens displayed large ocular dominance shifts to the open eye. However, the drug-treated animals also showed large ocular dominance shifts, despite having a mean cortical NA content of less than 12% of control (Table 2 and Fig. 3B). In fact, one kitten (K119) with 10 days of monocular deprivation displayed a complete ocular dominance shift with less than 1% of control cortical NA (Table 2). We conclude that in the case of animals treated neonatally with 6-OHDA, a large depletion of neocortical NA is not a sufficient condition for reducing plasticity.



**Fig. 3** HPLC confirmation of cortical catecholamine depletion by both the minipump delivery and neonatal injection of 6-OHDA. **A**, NA in the 6-OHDA treated tissue of the minipump series was only 21% of the same animal control hemispheres. DA levels were less affected. **B**, The catecholamine depletion in the 6-OHDA treated kittens of the neonatal injection series, relative to littermate controls. The mean cortical NA level of these animals was less than 12% of control.

Thus although both intracortical and neonatal 6-OHDA delivery techniques effectively destroy the ascending noradrenergic innervation of cortex, only the intracortical procedure results in a loss of plasticity. One possible explanation

**Table 1** Minipump 6-OHDA series

| Physiology                   |                           |                         |            |         |              |              |       |                                   |
|------------------------------|---------------------------|-------------------------|------------|---------|--------------|--------------|-------|-----------------------------------|
| Kitten                       | Age at deprivation (days) | Age at recording (days) | Hemisphere | Drug    | No. of units | Binocularity |       |                                   |
| K134                         | 44                        | 51                      | Left       | 6-OHDA  | 13           | 0.69         |       |                                   |
|                              |                           |                         | Right      | Vehicle | 18           | 0.56         |       |                                   |
| K135                         | 51                        | 58                      | Left       | 6-OHDA  | 20           | 0.65         |       |                                   |
|                              |                           |                         | Right      | Vehicle | 28           | 0.36         |       |                                   |
| K136                         | 58                        | 65                      | Left       | Vehicle | 22           | 0.32         |       |                                   |
|                              |                           |                         | Right      | 6-OHDA  | 25           | 0.68         |       |                                   |
| K137                         | 71                        | 79                      | Left       | 6-OHDA  | 28           | 0.86         |       |                                   |
|                              |                           |                         | Right      | Vehicle | 16           | 0.69         |       |                                   |
| K138                         | 31                        | 38                      | Left       | 6-OHDA  | 27           | 0.78         |       |                                   |
|                              |                           |                         | Right      | 6-OHDA  | 27           | 0.70         |       |                                   |
| K140                         | 45                        | 52                      | Left       | Vehicle | 30           | 0.70         |       |                                   |
|                              |                           |                         | Right      | 6-OHDA  | 32           | *            |       |                                   |
| K143                         | 40                        | 47                      | Left       | Vehicle | 21           | 0.62         |       |                                   |
|                              |                           |                         | Right      | 6-OHDA  | 13           | 0.92         |       |                                   |
| Biochemistry <sup>†</sup>    |                           |                         |            |         |              |              |       |                                   |
| Control hemispheres          |                           |                         |            |         |              |              |       |                                   |
| Kitten                       |                           | K134                    | K135       | K136    | K137         | K143         | K140  | $\bar{x} \pm \text{s.e.m.}$       |
| Cortical NA (ng per g)       |                           | 135                     | 406        | 710     | 189          | 1169         | 303   | 485 $\pm$ 160                     |
| Cortical DA (ng per g)       |                           | 110                     | 163        | 71      | 228          | 325          | 118   | 169 $\pm$ 38                      |
| 6-OHDA hemispheres           |                           |                         |            |         |              |              |       |                                   |
| Kitten                       |                           | K134                    | K135       | K136    | K137         | K143         | K138L | K138R $\bar{x} \pm \text{s.e.m.}$ |
| Cortical NA (ng per g)       |                           | 17                      | 207        | 87      | 71           | 261          | 28    | 48 103 $\pm$ 36                   |
| Cortical DA (ng per g)       |                           | 63                      | 109        | 66      | 93           | 236          | 35    | 46 93 $\pm$ 26                    |
| NA (% of control hemisphere) |                           | 13                      | 51         | 12      | 36           | 22           | 6‡    | 10‡ 21 $\pm$ 6                    |

Physiological and biochemical data from kittens which had received minipump infusion of 6-OHDA for 1 week concurrently with monocular deprivation. In all cases the left eye was sutured closed. Also indicated are the number of units isolated from each hemisphere and the binocularity. Binocularity is defined as the number of cells in ocular dominance groups 2–6 divided by the total number of cells recorded. The binocularities of 6-OHDA and control hemispheres were different at the  $P < 0.0025$  confidence level. The 6-OHDA treated hemispheres had a mean of 21% of the NA level in the same animal control hemispheres (Fig. 3A).

\* The right hemisphere in K140 was not successfully depleted of catecholamines because of an obstruction in the pump tubing. This animal is not included in the ocular dominance histogram.

† The variations in catecholamine levels for the minipump-treated animals is possibly a result of the small size of the cortical samples (mean wet weight 102 mg). However, for each animal the 6-OHDA treated hemisphere always had an NA level of 50% or less of the control hemisphere level, a difference that is significant at the  $P < 0.05$  confidence level.

‡ Animal K138 received 6-OHDA in both hemispheres and its %NE was calculated relative to the mean NA level for all control hemispheres.



Table 2 Neonatal 6-OHDA series

## Physiology

| Kitten | 6-OHDA dose (mg per kg) | Age at deprivation (days) | Age at recording (days) | Hemisphere | No. of units | Binocularity |
|--------|-------------------------|---------------------------|-------------------------|------------|--------------|--------------|
| K115   | 2 × 100                 | 32                        | 42                      | Left       | 0            | —            |
|        |                         |                           |                         | Right      | 37           | 0.46*        |
| K116   | Control                 | 34                        | 44                      | Left       | 12           | 0.50         |
|        |                         |                           |                         | Right      | 29           | 0.21         |
| K119   | 2 × 200                 | 26                        | 36                      | Left       | 8            | 0.38         |
|        |                         |                           |                         | Right      | 15           | 0.20         |
| K122   | Control                 | 35                        | 45                      | Left       | 15           | 0.73         |
|        |                         |                           |                         | Right      | 30           | 0.40         |
| K127   | 2 × 200                 | 7                         | 54                      | Left       | 29           | 0.38         |
|        |                         |                           |                         | Right      | 18           | 0.17         |
| K129   | Control                 | 7                         | 57                      | Left       | 19           | 0.21         |
|        |                         |                           |                         | Right      | 20           | 0.30         |
| K130   | Control                 | 39                        | 49                      | Left       | 24           | 0.46         |
|        |                         |                           |                         | Right      | 14           | 0.29         |
| K131   | 2 × 200                 | 26                        | 36                      | Left       | 40           | 0.50         |
|        |                         |                           |                         | Right      | 0            | —            |
| K132   | Control                 | 28                        | 38                      | Left       | 18           | 0.44         |
|        |                         |                           |                         | Right      | 25           | 0.68         |
| K133   | 2 × 200                 | 30                        | 40                      | Left       | 6            | —†           |
|        |                         |                           |                         | Right      | 6            | —†           |

## Biochemistry

## Control animals

| Kitten                 | K114 | K116 | K120 | K122 | K129 | K130 | K132 | $\bar{x} \pm \text{s.e.m.}$ |
|------------------------|------|------|------|------|------|------|------|-----------------------------|
| Cortical NA (ng per g) | 138  | 77   | 111  | 192  | 81   | 80   | 58   | 105 ± 17                    |
| Cortical DA (ng per g) | 60   | 30   | 78   | 59   | 21   | 19   | 19   | 41 ± 9                      |

## 6-OHDA animals

| Kitten                 | K119 | K123 | K127 | K128 | K131 | K133 | $\bar{x} \pm \text{s.e.m.}$ |
|------------------------|------|------|------|------|------|------|-----------------------------|
| Cortical NA (ng per g) | <1   | <17  | <32  | <11  | 4    | 7    | <12 ± 5                     |
| Cortical DA (ng per g) | 28   | 17   | 16   | <2   | 41   | 22   | <21 ± 5                     |
| NA (% of control mean) | <1   | <16  | <20  | <10  | 4    | 7    | <12 ± 4                     |

Physiological and biochemical data from kittens injected as neonates with 6-OHDA and their littermate controls. In all cases, the left eye was sutured closed. Binocularity of 6-OHDA treated and control kittens was similar regardless of the hemisphere studied. The mean NA content of 6-OHDA treated kittens is less than 12% of control (Fig. 3B). Conventions are the same as in Table 1.

\* Ocular dominance data not included in Fig. 2 because biochemical determination of cortical catecholamines was not made in this kitten.

† Ocular dominance data not included in Fig. 2 because of a corneal lesion on the deprived eye.

is that intracortical infusion of 6-OHDA has a direct effect on plasticity which is independent of the NA depletion. Two lines of evidence argue against this possibility. First, 6-OHDA treatment prevents the ocular dominance shift even when the treatment is stopped 1 day before the monocular deprivation begins<sup>4</sup>. Second, Kasamatsu *et al.*<sup>5</sup> report that plasticity may be restored in 6-OHDA treated tissue by providing exogenous NA. It therefore seems that the conflicting results, from the minipump and neonatal kittens, must stem from differences in the timing and (or) duration of NA depletion produced by the two procedures. In the neonatal series, the cortical NA depletion was initiated during the first postnatal week and lasted for at least 1 month before physiological recording. In the minipump 6-OHDA series, the NA depletion was initiated at ages in excess of 1 month and lasted only for 7 days while the kitten was monocularly deprived. More experiments are required to pinpoint the critical difference. Nonetheless, we believe that the explanation which best fits the available data is, first, that a critical level of NA is normally required for the plastic response to MD and second, that mechanisms exist to compensate for the chronic loss of cortical NA in kittens treated with 6-OHDA at birth.

One compensatory mechanism for chronic denervation is receptor supersensitivity<sup>18</sup>. However, while it is known that cortical beta receptors proliferate in response to locus coeruleus lesions<sup>19</sup>, the evidence suggests that this response alone may not be adequate to compensate for a 90% loss of NA<sup>20</sup>. Recently, Harik *et al.*<sup>20</sup> have documented a compensation phenomenon, independent of receptor supersensitivity, that occurs 4 weeks after locus coeruleus lesions in adult rats. The

mechanism for this recovery of function remains obscure. Whatever the mechanism, the idea of cortical compensation for chronic depletion of NA offers an explanation that is consistent with available data. This idea predicts that deficits in cortex stemming from early destruction of the noradrenergic innervation would only be transient.

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## Vitamin A is essential for photoperiodic induction of diapause in an eyeless mite

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The perception of photoperiod by insects and mites requires a photoreceptor which is probably located in the brain<sup>1-5</sup>. Recently, the functional involvement of carotenoids in the photoperiodic reaction of two mite species has been demonstrated using techniques based on mutant studies and nutritional studies respectively<sup>6-9</sup>. In dietary studies with insects, evidence for the participation of carotenoids in the photoperiodic induction of diapause has been found in one moth species<sup>9</sup>, whereas carotenoid deprivation has no effect in another moth<sup>10</sup> and a butterfly<sup>11</sup>. Here we show that vitamin A as well as carotenoids with provitamin A function, but not vitamin A acid, restores the photoperiodic reaction in an eyeless predaceous mite which has lost all sensitivity to the photoperiod after having been reared for more than one generation on pollen of broad bean, which constitutes a completely carotenoid-free diet. This study suggests that a rhodopsin-like pigment functions as the photoreceptor for the photoperiodic induction of diapause in these mites.

The phytoseiid mite *Amblyseius potentillae* is a predator of spider mites in apple orchards in the Netherlands, but in the laboratory it may be successfully reared on a vegetarian diet of pollen. No differences in growth and reproduction were observed between mites fed on spider mite prey and mites reared on pollen of broad bean, *Vicia faba*. Diet may influence the ability of these mites to enter diapause, however, and mites fed on eggs of albino spider mites or on broad bean pollen could be induced to diapause only if the respective diets were supplemented with  $\beta$ -carotene<sup>8,12</sup>. The fact that broad bean pollen constitutes a carotenoid-free diet for these mites made it possible to determine more specifically which carotenoids or carotenoid derivatives restore the ability to diapause in mites reared on the pollen diet.

Table 1 shows that addition of  $\beta$ -carotene, 3-hydroxyechinenone and vitamin A acetate to the pollen diet leads to the induction of full diapause under the short-day regime, whereas the addition of astaxanthin or vitamin A acid results in non-diapause comparable to that found in controls reared on pollen without any additional pigments. In long-day conditions, no diapause was found with any of the diets tested (Table 1). Apparently vitamin A and carotenoids with provitamin A function (that is, carotenoids which may be converted to vitamin A because at least one half of the molecule has the  $\beta$ -carotene configuration) relieve the deficiency of the diet with regard to photoperiodic induction, whereas astaxanthin and vitamin A acid cannot. As the reverse biosynthetic route, leading from vitamin A to carotenoids, has never been found in animals<sup>13,14</sup>, these results provide evidence for the functional involvement of vitamin A in the photoperiodic induction of diapause in these mites. It seems that mites deficient in vitamin A have become blind to the photoperiod and behave as if reared in continuous darkness. From these results we conclude that vitamin A, or more probably vitamin A aldehyde in the form of a membrane-bound rhodopsin-like complex, may well be the photoreceptor for the photoperiodic induction of diapause in these mites.

Carotenoids are known to be involved in the pigmentation of insects and mites<sup>15,16</sup>, and for some insects, growth-

**Table 1** Diapause incidence in *A. potentillae* fed diets with and without carotenoids or vitamin A

| Pigment supplement        | LD 8:16 |       | LD 16:8 |       |
|---------------------------|---------|-------|---------|-------|
|                           | %       | (n)   | %       | (n)   |
| None                      | 0       | (48)  | 0       | (33)  |
| $\beta$ -Carotene         | 98      | (147) | 0       | (127) |
| 3-Hydroxyechinenone (rac) | 100     | (103) | 2       | (60)  |
| Astaxanthin (rac)         | 0       | (44)  | 0       | (33)  |
| Vitamin A acetate         | 100     | (167) | 1       | (137) |
| Vitamin A acid            | 4       | (131) | 1       | (140) |

Mites were kept on rectangles of black plastic (8 × 15 cm), surrounded by a barrier of moist filter paper<sup>26</sup>. Cultures were supplied with pollen of *V. faba* as the only source of food.  $\beta$ -Carotene, 3-hydroxyechinenone (3-hydroxy-4-keto- $\beta$ -carotene), astaxanthin (3,3'-dihydroxy-4,4'-diketo- $\beta$ -carotene), vitamin A acetate (retinol) and vitamin A acid (retinoic acid) were added in crystalline form (5% w/w) and mixed with the pollen. Experiments were performed at 18 °C in climatic rooms in short-day (light/dark, 8:16 h) (LD 8:16) and long-day (LD 16:8) conditions respectively. *A. potentillae* exhibits a facultative reproductive diapause, induced by short-day photoperiods experienced during the immature stages. The critical daylength of our Dutch strain of mites is 14 h; diapause is absent in mites reared in continuous darkness. The stock culture was kept on a diet of broad bean pollen for several generations. Mites were reared on the diets from the egg stage onwards; all females mated during the first few days of adult life. Diapause incidence (%) was determined 2 weeks after the last moult. Complete reproductive arrest of individual female mites was taken as the criterion for diapause. n, Number of females per test.

promoting effects of carotenoids and vitamin A have been established<sup>17</sup>. Vitamin A is essential for vision in insects<sup>13,18</sup> and probably in mites also, although it has not yet been demonstrated in a mite species. That vitamin A is involved in photoperiodic induction seems to be a novel function for the vitamin in an animal. Moreover, as *A. potentillae* is an eyeless mite, photoperiodic photoreception must occur extraretinally, as has also been shown to be the case in several insect species<sup>19</sup>. An interesting parallel between the vision process and photoperiodic induction is that vitamin A is effective but vitamin A acid is not. Any speculations about the primary photoprocesses of the photoperiodic reaction would, however, be premature.

On the basis of feeding experiments<sup>20</sup> and action spectrum studies<sup>21</sup>, the photoreceptor for the entrainment of circadian rhythms in insects is believed to be not a carotenoid, but possibly a flavoprotein<sup>22</sup>. Although it is often assumed that the photoreceptors for photoperiodic induction and for the entrainment of circadian rhythms are identical, the circadian nature of the photoperiodic 'clock' in insects and mites has not been established irrefutably<sup>23</sup>. It is possible, therefore, that the photoreceptors for photoperiodism and for circadian rhythmicity are different, a rhodopsin-like pigment being the photoreceptor for the former reaction and a flavoprotein for the entrainment of circadian rhythms. Another possibility would be that the photoreceptor consists of both vitamin A and flavin<sup>24</sup>, although only one pigment was thought to be involved in the photoperiodic regulation of polymorphism in an aphid, a conclusion reached on the basis of extensive studies of action spectra<sup>25</sup>. As an action spectrum alone cannot provide conclusive data as to the nature of the photoreceptor<sup>24,25</sup>, nutritional studies may prove a valuable tool for the identification of extraretinal photoreceptor pigments in both insects and mites. Generalizations with regard to the function of vitamin A in the perception of photoperiod will, however, have to await the results of dietary studies with other arthropod species.

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## *Drosophila* mutants reveal two components of fast outward current

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The gating of potassium ion channels has been shown to be dependent on voltage<sup>1</sup> or Ca<sup>2+</sup> ions<sup>2,3</sup> or both<sup>4,5</sup>. A fast transient potassium current (sometimes denoted  $I_A$ ) is found in a wide variety of animals<sup>6–14</sup>. The Ca<sup>2+</sup>-sensitivity of this early outward current has been a matter of dispute as reports from different systems have indicated complete insensitivity<sup>6–8</sup>, marked sensitivity<sup>10–12</sup> or only partial sensitivity<sup>13,14</sup>. It is possible that there are two distinct early outward current systems, one Ca<sup>2+</sup>-sensitive and the other not. Thus, the reports of partial Ca<sup>2+</sup>-sensitivity would indicate the presence of both systems in the membrane. I now report that in the adult *Drosophila* flight muscles, the transient outward current is also partially Ca<sup>2+</sup>-sensitive. The hypothesis that two separate currents are present is strengthened by the discovery that in several mutants of the X-linked *Shaker* locus ( $Sh^{KS133}$ ,  $Sh^{102}$  and  $Sh^{K0120}$ ; refs<sup>15–17</sup>) the Ca<sup>2+</sup>-independent component ( $I_{Aci}$ ) is absent with only the Ca<sup>2+</sup>-dependent component ( $I_{Acd}$ ) remaining. Hence, the mutations seem to delete one of two separate current systems. An alternative hypothesis is that only one channel type is present that can be modified by mutation to be totally Ca<sup>2+</sup>-dependent.

The techniques for voltage clamping the *Drosophila* dorsal longitudinal flight muscles (DLM) have been reported previously<sup>17–19</sup>. In adult *Drosophila* flight muscle, in addition to fast transient outward current, there are two other currents, a transient inward Ca<sup>2+</sup> current ( $I_{Ca}$ ) and a delayed outward current ( $I_K$ ). The composite response of these currents to a voltage-clamp step pulse is shown in Fig. 1a,b. Note also Fig. 2a where the currents are shown on a faster time scale. In normal saline (see Fig. 1 legend) the response of mutant  $Sh^{KS133}$  muscles fibres (Fig. 1a) is similar to the wild-type response (Fig. 1b); all currents appear to be present. The prominent early peak of outward current is seen in both mutant and wild-type. However, mutant fibres have rather less peak outward current than wild-type fibres in normal, Ca<sup>2+</sup>-containing saline. Peak current at +10 mV was measured in 10  $Sh^{KS133}$  and 10 wild-type fibres and normalized with respect to  $I_K$  (note that  $I_K$  is not affected by these mutations<sup>17</sup>). In  $Sh^{KS133}$  the value was  $0.88 \pm 0.11$  compared with  $1.06 \pm 0.09$  ( $\pm$  s.d.) in

wild-type. (Student's *t*-test showed a greater than 99.5% probability of statistical significance.)

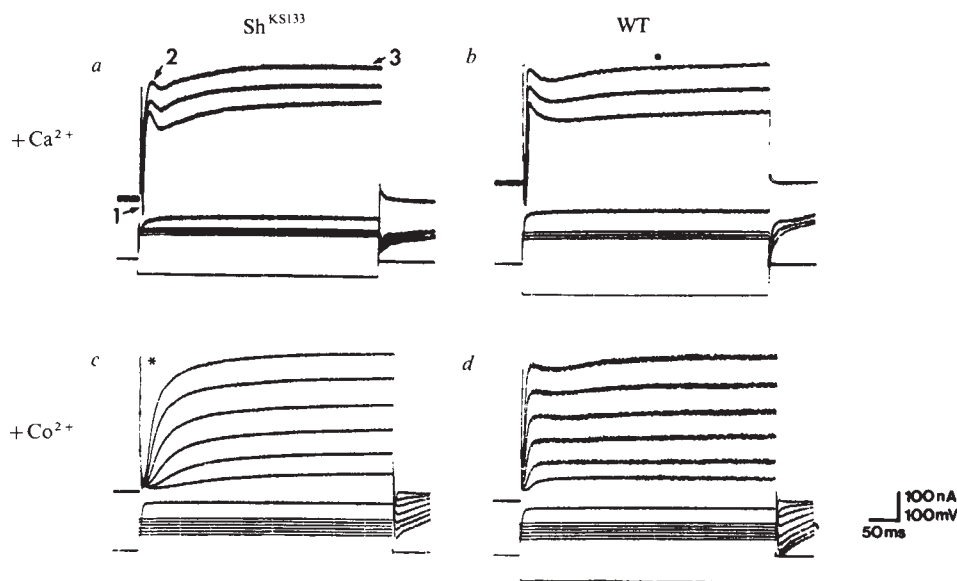
In addition to a difference in the amount of peak outward current present, another difference between mutant and wild-type became evident when  $I_{Ca}$  was eliminated from these preparations. The inward Ca<sup>2+</sup> current (arrow 1 in Fig. 1a) may be eliminated by replacing normal saline with Ca<sup>2+</sup>-free saline containing either Co<sup>2+</sup> (20 mM) or EGTA (5 mM). This treatment requires up to 20 min to eliminate  $I_{Ca}$  from some DLM fibres because of limited diffusion deep in the muscle stack. When the Ca<sup>2+</sup> current was eliminated in either of these ways, all the early peak outward current was also eliminated in mutant muscle fibres (Fig. 1c) but not in wild-type fibres (Fig. 1d). In Fig. 1c only the more slowly activating delayed outward current remained after treatment, while in Fig. 1d the early peak outward current was still prominent. Over 100 wild-type fibres were examined in which  $I_{Ca}$  was completely absent; a prominent early peak outward current was present in all of them. In contrast, in all of 50 mutant fibres where  $I_{Ca}$  had been eliminated, early peak outward current was also absent. The elimination of peak outward current in the mutant is reversible. When EGTA or Co<sup>2+</sup> is washed out and replaced with Ca<sup>2+</sup>-containing saline, the peak outward current returns with the return of  $I_{Ca}$ .

The calcium requirement of early peak outward current in mutants was investigated further by substituting Ba<sup>2+</sup> for Ca<sup>2+</sup> in the saline. It is known that Ba<sup>2+</sup> can carry current through  $I_{Ca}$  channels<sup>20–22</sup> but it does not activate Ca<sup>2+</sup>-dependent potassium currents<sup>3</sup>. It was of interest, therefore, to determine whether Ba<sup>2+</sup> could activate the transient outward current in the mutant. It was found that Ca<sup>2+</sup> itself is necessary. When Ba<sup>2+</sup> was substituted for Ca<sup>2+</sup> in the saline, a large inward current was present in both wild-type and mutant cells, but while a transient outward current was prominent in wild-type it was absent from mutant cells.

Although in mutant fibres all of the transient outward current is calcium-dependent, in wild-type both calcium-dependent and independent components were found to be present. A precedent exists for this in sheep cardiac Purkinje cells<sup>13</sup>, in which there are two transient outward currents: a calcium-activated current and a calcium-independent current. Only the calcium-independent component is blocked by aminopyridine drugs. This is also the case in the wild-type *Drosophila* DLM, where aminopyridine drugs block  $I_{Aci}$  but not  $I_{Acd}$ . Thus, these drugs provide a means of isolating  $I_{Acd}$  in adult wild-type fibres.

The effect of 3-aminopyridine (3-AP, 3 mM) on wild-type fibres is illustrated in Fig. 2. Figure 2a shows a wild-type preparation in normal saline before the addition of 3-AP; a large transient outward current was present. Forty minutes after the addition of 3-AP; a portion of peak outward current had disappeared (Fig. 2b). However, when Ca<sup>2+</sup> was removed and replaced with Co<sup>2+</sup> still in the presence of 3-AP, the remaining early peak outward current was completely abolished when  $I_{Ca}$  was eliminated (Fig. 2c). Doing the experiment in the reverse order allowed the Ca<sup>2+</sup>-dependent component to be eliminated while the Ca<sup>2+</sup>-independent component remained. Figure 2d shows the Ca<sup>2+</sup>-independent current present in wild-type fibres in Co<sup>2+</sup>-saline but with no 3-AP. This current was completely eliminated as in Fig. 2c when 3-AP was added. Hence, there are two separable components of early peak outward current in the wild type:  $I_{Acd}$  which is isolated by using 3-AP, and  $I_{Aci}$  which is isolated by Co<sup>2+</sup>.  $I_{Acd}$  in mutant fibres is similar in all respects to  $I_{Acd}$  of wild-type fibres, it is not blocked by aminopyridine drugs (3- or 4-aminopyridine (3 mM) or 3, 4-diaminopyridine (1 mM)). The relative amount of  $I_{Acd}$  and  $I_{Aci}$  in a wild-type fibre is difficult to determine because overlapping currents make quantitative analysis imprecise. However, the algebraic sum of  $I_{Acd}$  and  $I_{Aci}$  when each is viewed separately seems to exceed the total amount of peak outward current when both are present. This could be a signal that the two currents are not carried by independent systems. Thus, such possibilities as the two currents being carried by different states

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**Fig. 1** Current responses to voltage-clamp step pulses in *Sh<sup>KSI33</sup>* (a, c) and wild-type, WT (Canton-S strain) (b, d). a, b, Composite current responses in mutant (a) and wild-type (b) cells in  $\text{Ca}^{2+}$ -containing saline (see below). Current is shown in upper traces, voltage in lower traces. Arrow 1 =  $I_{\text{Ca}}$ ; arrow 2 = peak of transient outward current; arrow 3 = non-inactivating outward current,  $I_{\text{K}}$ . c, d, Current responses of mutant (c) and wild-type (d) cells in saline containing  $\text{Co}^{2+}$  to block  $I_{\text{Ca}}$ . The asterisk in c marks the point where the transient outward current would peak if it were present. Holding potential,  $-80$  mV. The maximum voltage step in each figure is to  $\sim +20$  mV. Lower voltage steps are at  $\sim 10$  mV intervals. The composition of saline was (mM): NaCl, 128; KCl, 4.8;  $\text{CaCl}_2$ , 1.8 or  $\text{CoCl}_2$ , 20; Tris-HCl, 2, pH 7.0. All experiments were  $4^\circ\text{C}$ .

of the same channel, or a competition between two channels for a shared factor, will have to be investigated.

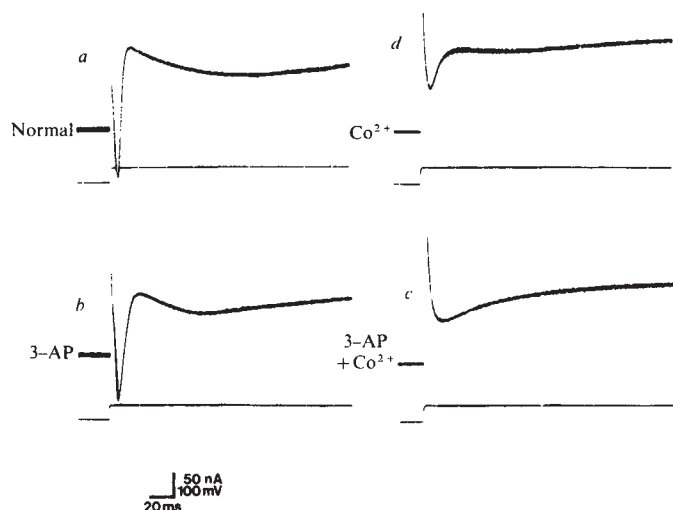
$I_{\text{Aci}}$  and  $I_{\text{Acd}}$  can also be distinguished by their significantly different rates of recovery from inactivation. I demonstrated this by testing recovery from inactivation in a standard double-pulse experiment. In Fig. 3,  $I_{\text{Aci}}$  was isolated in a wild-type fibre by  $\text{Co}^{2+}$  treatment (Fig. 3a),  $I_{\text{Acd}}$  was isolated in the wild-type by 3-AP treatment (Fig. 3b), and  $I_{\text{Acd}}$  is shown in a *Sh<sup>KSI33</sup>* fibre (Fig. 3c). In each case a voltage-clamp step pulse of 200 ms duration was applied to the membrane, and then reapplied after an interpulse interval of 1.2 s. In Fig. 3a,  $I_{\text{Aci}}$  recovered almost completely during the 1.2 s interval; the superimposed traces of the first and second pulses are almost identical. In contrast, for  $I_{\text{Acd}}$ , both in wild-type (Fig. 3b) and *Sh<sup>KSI33</sup>* (Fig. 3c),  $I_{\text{Acd}}$  showed little or no recovery during the same interpulse interval; in the second pulse, inward current dominated under the prominent peak of early outward current evoked by the first pulse. Whereas the three currents  $I_{\text{Ca}}$ ,  $I_{\text{Acd}}$  and  $I_{\text{K}}$  were all separately resolvable during the first-pulse, only  $I_{\text{Ca}}$  and  $I_{\text{K}}$  were evident during the second pulse. Thus,  $I_{\text{Acd}}$  in both wild-type and mutant fibres shows a similar slow recovery that is markedly different from the more rapid recovery of  $I_{\text{Aci}}$  in wild-type.

The multiplicity of simultaneous currents when  $I_{\text{Acd}}$  is observed make measurements of activation and inactivation imprecise. However, it seems that  $I_{\text{Acd}}$  inactivates more rapidly than  $I_{\text{Aci}}$  because the dip between the early outward peak of current and the rising phase of  $I_{\text{K}}$  was more pronounced for  $I_{\text{Acd}}$  in mutant and AP-treated wild-type fibres than for  $I_{\text{Aci}}$  in  $\text{Co}^{2+}$ -treated wild-type fibres. The activation of  $I_{\text{Aci}}$  and  $I_{\text{Acd}}$  also differs. The activation of the former in adult fibres did not decline at high voltage steps up to the limit tested ( $+30$  mV), whereas the activation of  $I_{\text{Acd}}$  followed the activation curve of  $I_{\text{Ca}}$  which declines at high voltages<sup>19</sup>. This was evident in preparations where  $I_{\text{Ca}}$  was reduced by  $\text{Co}^{2+}$  treatment; before  $I_{\text{Ca}}$  was totally eliminated  $I_{\text{Acd}}$  could be evoked at low voltages where  $I_{\text{Ca}}$  was largest ( $-25$  to  $-10$  mV), but not at higher voltages where  $I_{\text{Ca}}$  was very small.

In a previous developmental study of ion currents in the wild-type *Drosophila* DLM, it was shown that  $I_{\text{A}}$  begins to appear midway through pupal development, but that  $I_{\text{Ca}}$  only appears at the time of adult eclosion<sup>18</sup>. Thus,  $I_{\text{A}}$  in the pupa is the  $\text{Ca}^{2+}$ -independent type which is blocked by 3-AP<sup>17</sup>. In a study of mutant *Sh<sup>KSI33</sup>* animals, it was shown that  $I_{\text{A}}$  never developed in mutant fibres during the pupal period, but was present in some of the adult fibres<sup>17</sup>. From the results presented here, it is clear that this current is  $\text{Ca}^{2+}$ -dependent and, thus, is seen only in those adult fibres where  $I_{\text{Ca}}$  is present.

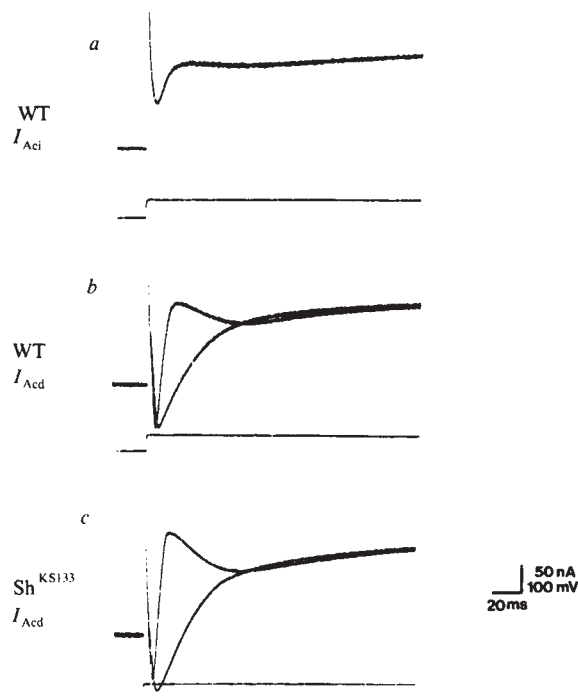
*Shaker* mutations that delete  $I_{\text{Acd}}$  from muscles also affect neurones<sup>15,16</sup>. The total dependence of transient outward current in mutants on  $\text{Ca}^{2+}$  influx can explain the aberrant spike propagation and synaptic transmission in *Shaker* mutants. In *Shaker* axons, it was found that spike repolarization was abnormally slow whether  $\text{Ca}^{2+}$  was available or not<sup>16</sup>. This is expected as axons have little or no  $\text{Ca}^{2+}$  current and therefore no  $\text{Ca}^{2+}$  influx to activate the  $\text{Ca}^{2+}$ -dependent  $I_{\text{A}}$  channels (if, indeed, these channels are present in axons). Conversely, it was found that repolarization of the presynaptic terminal, where there are many  $\text{Ca}^{2+}$  channels, was strongly dependent on  $\text{Ca}^{2+}$  concentrations<sup>15</sup>.

Another mutation at the *Shaker* locus, *Sh<sup>5</sup>*, is unlike these other *Shaker* mutations where  $I_{\text{A}}$  is absent in the pupa. In *Sh<sup>5</sup>*  $I_{\text{A}}$  is present in pupae but its kinetics are altered from wild-type; the mutation speeds up both inactivation and the recovery from inactivation<sup>17</sup>. Thus, on biophysical criteria, the *Sh<sup>5</sup>* mutation



**Fig. 2**  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent current components in wild-type DLM fibres separated by 3-AP and  $\text{Co}^{2+}$  treatment. Current responses (upper traces) are shown to voltage-clamp step pulses (lower traces). a, Wild-type in normal saline. b, Wild-type 40 min after addition of 3-AP (3 mM); the peak outward current declined but remained prominent. c, Wild-type after the replacement of  $\text{Ca}^{2+}$  with  $\text{Co}^{2+}$  (20 mM) in 3-AP saline, all the early peak outward current was abolished. d, Wild-type fibre 40 min after replacement of  $\text{Ca}^{2+}$  with  $\text{Co}^{2+}$  (no 3-AP present). 3-AP added to this preparation completely eliminated all early peak outward current (as in c). Holding potentials,  $-70$  mV.





**Fig. 3** Recovery from inactivation as shown by double-pulse experiments. *a*,  $I_{Aci}$  in a  $Co^{2+}$ -treated wild-type fibre. *b*,  $I_{Acd}$  in a 3-AP-treated wild-type fibre. *c*,  $I_{Acd}$  in a  $Sh^{KS133}$  fibre with rml saline. In each case superimposed traces are shown where a 200-ms step pulse was applied to the membrane and then reapplied after an interpulse interval of 1.2 s. In *a*, the superimposed traces are almost identical, showing almost complete recovery of  $I_{Aci}$  during the interpulse interval. In *b* and *c* only  $I_{Ca}$  and  $I_K$  are evident during the second pulse, showing little or no recovery of  $I_{Acd}$  during the interval. Holding potential,  $-70$  mV.

is distinct from the other *Shaker* mutations. However, genetic recombination experiments show that the mutations  $Sh^{KS133}$  and  $Sh^5$  are either within the same gene or are in genes which are very close to one another. Thus, the possibility exists that  $Sh^5$  and  $Sh^{KS133}$  both affect the same channel component;  $Sh^5$  may cause a subtle change in the component while  $Sh^{KS133}$  removes its biological activity altogether. Alternatively, the two mutations may affect different components of the channel encoded by closely linked genes.

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## Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants

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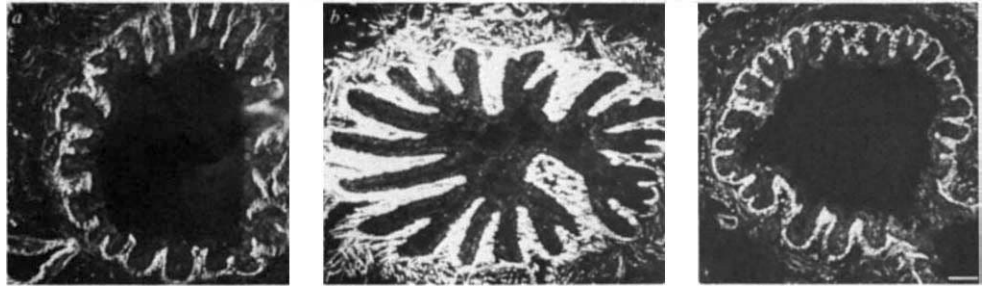
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The mucosa of the trachea and bronchi is very sensitive to various types of stimuli. Thus, cigarette smoke as well as mechanical or chemical irritation induce local mucosal reactions and bronchial smooth muscle spasm via sensory reflexes<sup>1–4</sup>. However, only the chemical transmitters involved in these local or vago-vagal reflexes acting to produce bronchoconstriction are known, while the mediator of the vasodilation remains to be established<sup>5</sup>. Unmyelinated sensory neurones of the C-fibre group have been associated with 'neurogenic inflammation', that is, increased vascular permeability and antidromic vasodilation in the skin<sup>5,6</sup>. These responses are abolished after pretreatment with capsaicin, the pungent agent of red peppers<sup>5,6</sup>. Simultaneously, there is a loss of substance P-immunoreactive nerves<sup>7</sup>, which may explain the long-term effect of capsaicin, as substance P normally increases vascular permeability and blood flow<sup>6</sup>. Capsaicin-sensitive, substance P-immunoreactive neurones of vagal sensory origin have also recently been found in the lower respiratory tract<sup>8</sup>. Furthermore, preliminary observations suggest that an increase in vascular permeability occurs in the tracheal mucosa on vagal nerve stimulation<sup>9–10</sup>. The capsaicin-sensitive afferents of vagal origin are probably also involved in local regulation of bronchial smooth muscle tone<sup>8,11</sup>. We report here that cigarette smoke as well as light mechanical or local chemical (ether, formalin, histamine, bradykinin or capsaicin) irritation and vagal nerve stimulation induced a subepithelial oedema in the rat trachea and bronchial tree, as indicated by extravasation of Evans blue. The increase in vascular permeability induced was markedly reduced or abolished in capsaicin-pretreated animals, where substance P-containing C-fibre afferents in the respiratory tract had degenerated. Substance P had a potent direct stimulatory effect on vascular permeability in the airways. Irritation of the respiratory tract mucosa seems to activate capsaicin-sensitive neurones, which via local axon reflexes induce an interstitial oedema probably by releasing substance P. Capsaicin pretreatment induces a long-lasting desensitization of the airways to various exogenous and anaphylactic irritants. These findings may give new aspects of the pathophysiology and treatment of hyperreactive airways in man.

Vascular permeability to serum albumin in the trachea was studied as an indication of mucosal reactivity in male Sprague-Dawley rats (250–300 g)<sup>12</sup>. Both normal animals and rats treated subcutaneously (s.c.) with capsaicin<sup>5,6</sup> (Sigma) 50 mg per kg on the second day of life were investigated. Newborn control animals were treated with solvent without capsaicin<sup>5,6,13</sup>. Experiments were performed 3 months later.

Exposure to smoke from one cigarette induced a tracheal oedema as indicated by a clearcut increase in Evans blue extravasation (Table 1). This smoke-induced permeability increase was abolished after capsaicin pretreatment (Table 1). When animals were exposed to ether, they showed an excited adverse and avoidance behaviour, and after 5 min of ether anaesthesia a significant, atropine-resistant Evans blue extravasation was observed in the trachea. In contrast, capsaicin-pretreated animals exhibited hardly any avoidance behaviour on ether exposure and no permeability increase was observed. Moreover, the time between ether exposure and anaesthesia in these animals was only  $29 \pm 3$  s ( $n = 5$ ) compared with  $52 \pm 5$  s ( $n = 5$ ;  $P < 0.01$ ) in control animals. Local injections of capsaicin, histamine, bradykinin, formalin or substance P into the

**Fig. 1** Fluorescence micrograph of bronchi from the rat lung (a-c) after i.v. injection of Evans blue 20 mg per kg. a, Control animal; b, c, animals subjected to unilateral vagus nerve stimulation (10 V, 10 Hz, 0.2 ms) for 5 min. a, b, Normal rats; c, adult animal pretreated with capsaicin (50 mg per kg, s.c.) at day 2 of life. Following rinsing with 40 ml saline, thin (2 mm thick) slices of the lung were dissected out and immersion-fixed in ice-cold phosphate buffer (0.1 M, pH 7.4) containing 10% formalin for 2 h. Following 24 h rinsing in a solution of phosphate-buffered saline containing 5% sucrose, 10- $\mu$ m cryostat sections were cut and mounted in glycerin-phosphate-buffered saline (3:1 v/v). They were then examined in a Zeiss fluorescence microscope equipped with a Scott BG 12 filter<sup>12</sup>. In this illumination Evans blue extravasation (b) is seen as a bright red fluorescence (that is, white in this black and white micrograph). The fluorescence due to Evans blue leakage was strongest in the subepithelial layer of the respiratory mucosa (b). The subepithelial oedema sometimes almost obliterated the bronchial lumen (b). Frequently, a patchy red fluorescence was seen, which due to adherence to connective tissue components. Similar types of subepithelial, bronchial and tracheal Evans blue extravasation, although of varying degrees, were also seen after cigarette smoke, substance P, capsaicin, histamine, bradykinin, formalin, ether or mechanical irritation. Autofluorescent connective tissue components can be seen close to the respiratory epithelium in a and c. Scale bar, 50  $\mu$ m.



tracheal lumen caused Evans blue extravasation which was significantly greater than the small effect induced by saline (Table 1). The vascular leakage induced by capsaicin, histamine, bradykinin and formalin but not that of substance P was significantly reduced or abolished in animals pretreated neonatally with capsaicin (Table 1). Local anaesthesia by lidocaine also reduced the effect of capsaicin but not that of substance P (Table 1). Local injections of various other peptides, which have been reported to be located in spinal neurones sensitive to capsaicin pretreatment (see ref. 13)—vasoactive intestinal polypeptide, somatostatin, bombesin and cholecystokinin (8 nmol of each)—did not cause Evans blue extravasation in the trachea ( $n = 4$  for each group). Intubation with a wide tube

caused a strong tracheal extravasation, but this reaction was not significantly changed in capsaicin-pretreated animals (Table 1). The more moderate mechanical irritation caused by a thin soft catheter induced an Evans blue extravasation which was reduced by local lidocaine administration as well as by neonatal capsaicin pretreatment. Electrical stimulation of the right vagus nerve below the nodose ganglion induced an atropine-resistant, subepithelial extravasation which was reduced by neonatal capsaicin pretreatment. Electrical stimulation of the right vagus nerve induced an atropine-resistant, subepithelial extravasation in the lower half of the trachea and in the stem and hilus bronchi on both sides as well as in bronchioli of both lungs (Fig. 1). Also, this response was dependent on capsaicin-sensitive nerves

**Table 1** Effect of various forms of irritation on tracheal mucosal reactivity as measured by Evans blue extravasation

|                             |           | Evans blue extravasation* ( $\bar{X} \pm$ s.e.m., $n$ in parentheses) |                         |                         |                          |
|-----------------------------|-----------|-----------------------------------------------------------------------|-------------------------|-------------------------|--------------------------|
|                             |           | Normal animals                                                        | Atropine                | Lidocaine               | Capsaicin                |
| Control                     |           | $8 \pm 1$ (4)                                                         | ND                      | ND                      | ND                       |
| Cigarette smoke             |           | $42 \pm 5$ (4)                                                        | ND                      | ND                      | $5 \pm 1$ (4) -88%‡      |
| Ether                       |           | $48 \pm 8$ (5)                                                        | $55 \pm 13$ (4) +15% NS | ND                      | $13 \pm 2$ (5) -73%‡     |
| Local saline                |           | $13 \pm 2$ (4)                                                        | ND                      | ND                      | ND                       |
| Capsaicin                   | 0.3 nmol  | $249 \pm 17$ (3)                                                      | ND                      | $72 \pm 23$ (5) -70%‡   | $20 \pm 1$ (4) -92%‡     |
| Histamine                   | 500 nmol  | $82 \pm 19$ (4)                                                       | ND                      | ND                      | $25 \pm 5$ (4) -70%‡     |
| Bradykinin                  | 10 nmol   | $98 \pm 11$ (5)                                                       | ND                      | ND                      | $26 \pm 5$ (5) -73%‡     |
| Formalin                    | 0.01%     | $189 \pm 12$ (4)                                                      | ND                      | ND                      | $12 \pm 3$ (4) -94%‡     |
| Substance P                 | 0.08 nmol | $67 \pm 4$ (9)                                                        | ND                      | ND                      | $59 \pm 4$ (6) -12% NS   |
| Substance P                 | 8 nmol    | $223 \pm 13$ (9)                                                      | ND                      | $226 \pm 38$ (5) +1% NS | ND                       |
| Intubation                  |           | $251 \pm 45$ (4)                                                      | ND                      | ND                      | $224 \pm 32$ (5) -11% NS |
| Light mechanical irritation |           | $175 \pm 14$ (6)                                                      | ND                      | $70 \pm 19$ (4) -60%‡   | $71 \pm 17$ (5) -59%‡    |
| Vagus stimulation           |           | $97 \pm 14$ (12)                                                      | $98 \pm 15$ (5) +1% NS  | ND                      | $14 \pm 3$ (4) -86%‡     |

For comparison, some animals were pretreated with atropine (0.5 mg per kg, i.v., 10 min before experiments), lidocaine (50  $\mu$ l of 20 mg ml<sup>-1</sup> into the tracheal lumen 5 min earlier) or neonatal capsaicin (50 mg per kg, s.c., on day 2 of life). Experiments were performed at the age of 3-4 months. The dye Evans blue, which rapidly binds to serum albumin, was injected i.v. (20 mg per kg) into conscious or sodium pentobarbital (40 mg per kg, intraperitoneally) anaesthetized animals to monitor changes in vascular permeability<sup>12</sup>. Following nerve stimulations, local injections, mechanical irritation or inhalation of smoke or ether for 5 min (see below), the animals were perfused with saline and a 1-cm piece of the trachea just proximal to the stem bronchi was excised and weighed. The Evans blue content of the trachea was extracted by formamide and quantified fluorometrically<sup>12</sup>. Control animals were treated as described below, but the irritating stimulus was omitted. Conscious rats were exposed to smoke from Kentucky reference 2R-1 type of cigarettes without filter in a Walton horizontal smoking machine<sup>27</sup>. The animals were first injected with Evans blue via the tail vein and then exposed to 12 puffs of smoke from one cigarette during 30 s intervals. Each puff of smoke was followed by 30 s of fresh air. In other experiments rats were exposed to an atmosphere saturated with ether until anaesthesia was induced. The time period from the onset of ether exposure until anaesthesia ('sleeping onset') was also measured. Local injection into the tracheal lumen of animals having an upright position at an angle of about 45° were performed just below the cricoid cartilage using a glass micropipette and a Hamilton syringe. 50  $\mu$ l of saline or irritants were then injected intratracheally 5 min before death. Some animals were intubated with a wide polyethylene catheter (diameter 2.4 mm) for 5 min. To study the effect of light mechanical irritation, the animals were first intubated with the tip just below the cricoid cartilage. After 15 min Evans blue was injected i.v. and a thin soft polyethylene tube (diameter 1.0 mm) was inserted through the lumen of the tracheal tube down to the tracheal bifurcation. Three rotations of this thin catheter were performed every minute for 5 min to irritate the tracheal mucosa. Electrical nerve stimulation was performed on the distal end of the cut right cervical vagus nerve (10 V, 0.2 ms, 10 Hz) for 5 min.  $n$ , Number of animals. ND, experiments not done. NS, not significant.

\* Values in ng Evans blue per mg wet weight of tissue.

†  $P < 0.01$  by Student's two-sample  $t$ -test.

‡  $P < 0.001$ .



(Table 1, Fig. 1). Pretreatment with compound 48/80 or mepyramine plus cimetidine<sup>6</sup> did not reduce the effects of vagal nerve stimulation nor of substance P and capsaicin ( $n = 5$  for each group). The mepyramine plus cimetidine pretreatment, however, abolished the extravasation induced by 500 nmol histamine.

Active or passive exposure to cigarette smoke is the most common form of irritation to the respiratory tract mucosa in man. Nicotine and formalin, which are present in cigarette smoke, are known to activate tracheobronchial C-fibre afferents<sup>1</sup>. In the present study, pretreatment with capsaicin abolished the effect of smoke as well as that of several other types of local irritants. Capsaicin is known to produce acute activation of afferent nerve endings of the C-fibre group in the respiratory tract<sup>1,14</sup>. These nerves have the characteristics of visceral nociceptor fibres, which are primarily activated during tissue damage and mediator release<sup>1</sup>. Neonatal capsaicin pretreatment induces selective degeneration of some afferent C-fibres<sup>15</sup> and a simultaneous loss of neuropeptides, including substance P, in the spinal cord<sup>7</sup>, nucleus tractus solitarius<sup>16</sup> and respiratory tract<sup>8</sup>. Substance P is a strong candidate for mediating the nervously induced increase in vascular permeability in the respiratory tract, as both the vagal effect and substance P response are blocked by a substance P antagonist<sup>17</sup>. The acute increase in vascular permeability caused by capsaicin is probably also mediated by substance P, because capsaicin has been shown to induce substance P release from the central branches of sensory neurones<sup>18,19</sup>. In accordance with this, local anaesthesia by lidocaine was found to inhibit the effect of capsaicin in the trachea. This suggests that a local axon reflex was necessary before capsaicin-mediated receptor activation (close to the epithelium) via substance P release induced a permeability response in the postcapillary venules<sup>20</sup>.

The effects of local anaesthetics on the skin-permeability response to capsaicin, however, have been contradictory<sup>21</sup>. It should be emphasized that lidocaine only reduced the capsaicin response by 60–70%, thus implying that capsaicin still released some substance P in the mucosa. Volatile anaesthetics such as ether activate C-fibres in the respiratory tract<sup>1</sup>, inducing local oedema as well as an initial bronchoconstriction<sup>11</sup>. Both these effects seem to be mediated by activation of capsaicin-sensitive afferent neurones. The lack of avoidance behaviour to ether in the capsaicin-pretreated rats, as well as the decrease in 'sleeping onset', probably reflects a loss of protective reflexes and subsequently an increased inhalation of the anaesthetic. Gentle mechanical stimulation also induces a capsaicin-sensitive increase in vascular permeability, suggesting a C-fibre activation<sup>1</sup>. Clinical experience also indicates that pretreatment with local anaesthetics reduces the oedema after mechanical manipulation of the laryngeal or tracheal mucosa. Tracheal intubation with a wide tube induces a profound Evans blue extravasation, probably caused by severe tissue damage, which seems to depend largely on mechanisms not involving capsaicin-sensitive neurones.

Histamine did not seem to be the final major mediator of the neurogenic inflammation response in the respiratory tract, as has been suggested for the skin<sup>6</sup>, but seemed rather to exert an indirect effect via activation of capsaicin-sensitive neurones. A similar mechanism was found for the effect of bradykinin in agreement with the findings from the skin<sup>22</sup>. Also, the bronchoconstrictor effect of intravenous (i.v.) histamine seems to a large extent to depend on stimulation of capsaicin-sensitive afferents<sup>11</sup>. Anaphylactic reactions have been found to stimulate afferent C-fibres in the respiratory tract<sup>23</sup>. This suggests that capsaicin-sensitive substance P-containing nerves may also be of importance for the pathophysiology of bronchial asthma.

In conclusion, the present findings illustrate that capsaicin pretreatment produces in the mucosa of the respiratory tract long-lasting desensitization to various mechanical and chemical irritants. Capsaicin seems to have potent actions on afferent nerves in human skin<sup>24,25</sup>. Furthermore, substance P-containing nerves are abundant in human airways (in preparation) and

capsaicin induces an acute constriction of human bronchi<sup>26</sup>. Capsaicin desensitization of the airway mucosa may thus provide new information on the pathophysiology and possibly also treatment of the mucosal oedema seen in hyperreactive airways and asthma.

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## Vasopressin stimulates formation of coated pits in rat kidney collecting ducts

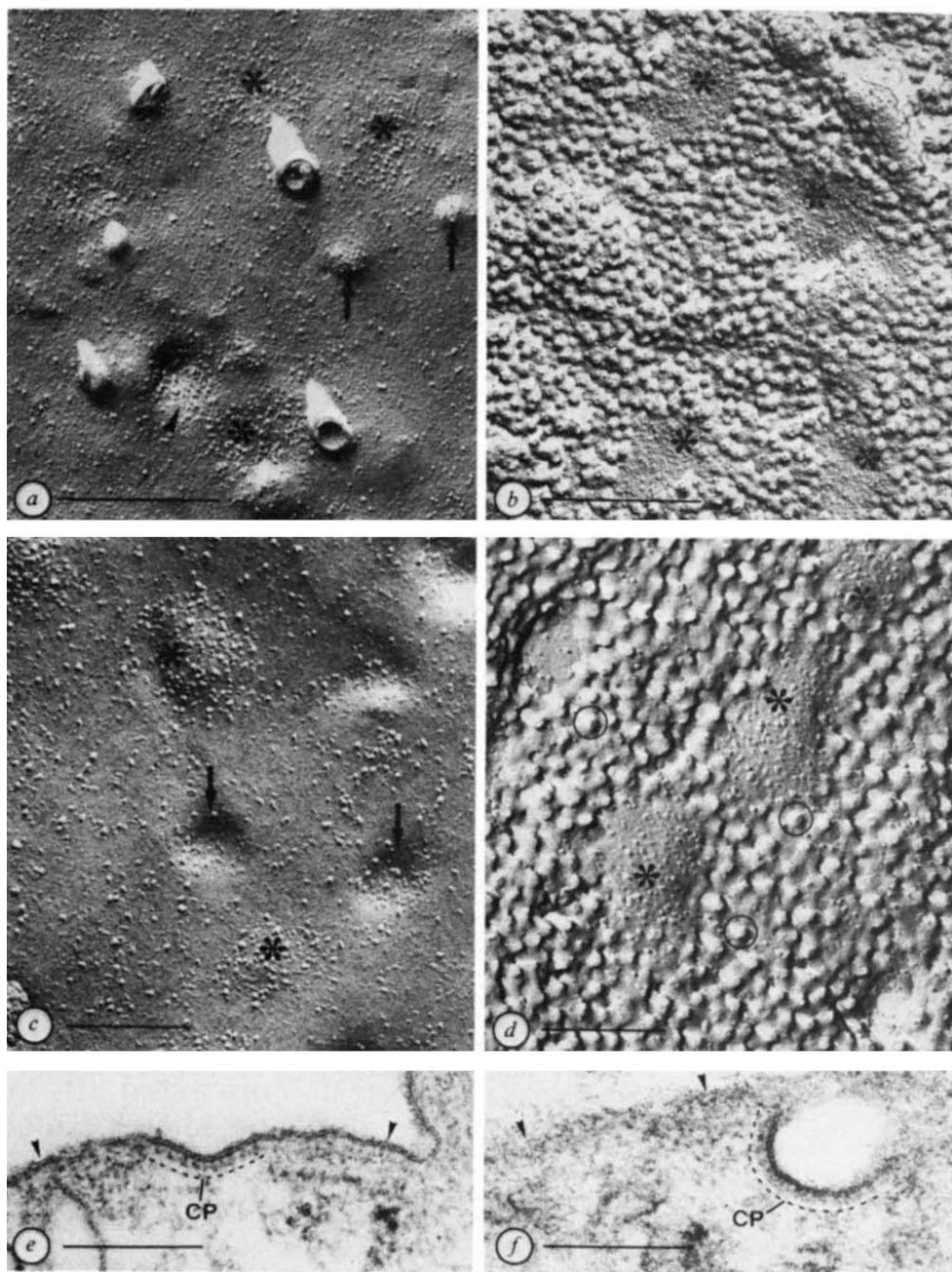
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The water permeability of collecting ducts is greatly increased by the antidiuretic hormone, vasopressin<sup>1</sup>. This permeability increase is associated with the appearance of intramembrane particle (IMP) clusters on the luminal plasma membranes of principal cells of the collecting duct epithelium<sup>2,3</sup>. IMP aggregates have also been related to an increase in water permeability of two other vasopressin-sensitive epithelia, the amphibian urinary bladder<sup>4</sup> and the amphibian epidermis<sup>5</sup>, and it has been proposed that these specialized membrane domains might represent specific water-permeable membrane patches, induced by the hormone in their respective epithelia. Using a cytochemical probe for membrane cholesterol, filipin<sup>6</sup>, we show here that the membrane patches in rat kidney are selectively devoid of filipin-sterol complexes and that when identified in thin sections, they have a cytoplasmic bristle coat: both of these features are characteristics of coated pits<sup>7</sup> which, in other systems, are involved in receptor-mediated endocytosis<sup>8</sup>. We also show that vasopressin induces the appearance of coated pits on collecting duct luminal membranes in Brattleboro homozygous rats, which have hereditary diabetes insipidus<sup>9</sup>.



**Fig. 1** *a*, Luminal plasma membrane P-face of a freeze-fractured collecting duct principal cell from water-deprived rat. Several clusters of intramembrane particles (some marked by asterisks) are present, as well as stubby microvilli which appear as small protrusions (arrows). One of the particle clusters (arrow-head) is present on an invaginating region of the membrane. Scale bar, 0.5  $\mu\text{m}$ . *b*, Same as *a* but from collecting duct exposed to filipin before freeze-fracturing. Filipin combines specifically with membrane cholesterol and this interaction results in the formation of filipin-sterol complexes in the plane of the membrane. These complexes appear as stud-like protuberances (or pits)  $\sim 25$  nm in diameter and they distort the structure of the membrane except in the regions of the particle clusters, which appear as ovoid patches (asterisks) free of complexes. Scale bar, 0.5  $\mu\text{m}$ . *c*, Same as *a* but showing the plasma membrane E-face. Two particle clusters are indicated with asterisks. On the E-face, microvilli appear as shallow depressions (arrows). Scale bar, 0.25  $\mu\text{m}$ . *d*, As for *c* but showing the E-face of a filipin-treated collecting duct. Filipin-sterol complexes (some are encircled) are selectively absent from the particle clusters (asterisks). Scale bar, 0.25  $\mu\text{m}$ . *e*, Thin section of the luminal plasma membrane of a collecting duct principal cell showing a coated pit (CP). The cytoplasmic bristle coat is clearly visible. The trilaminar membrane structure is preserved not only within the coated pit, but also on the rest of the apical membrane (arrowheads). Scale bar, 0.25  $\mu\text{m}$ . *f*, Same as *e* but from a filipin-treated collecting duct. At the level of a coated pit (CP), recognized by a distinct cytoplasmic bristle coat, the trilaminar membrane structure is preserved as no filipin-sterol complexes are formed in this membrane domain. The rest of the luminal plasma membrane is disrupted (arrowheads) by the filipin treatment and corresponds to the regions in which numerous filipin-sterol complexes can be seen in freeze-fracture. Scale bar, 0.25  $\mu\text{m}$ .



In the first series of experiments, adult male Wistar rats (300 g) were deprived of water for 24 h to induce the formation of IMP aggregates as described previously<sup>2,3</sup>. Rats were then anaesthetized with Nembutal (0.1 ml of a 50 mg ml<sup>-1</sup> solution per 100 g body weight) and were perfused through the left ventricle with 2.5% glutaraldehyde in 0.1 M phosphate buffer, following a short rinse (2 min) with phosphate-buffered isotonic saline. Kidneys were removed after fixation for 10 min and pieces from the inner stripe of the outer medulla and the inner medulla were fixed for a further 50 min by immersion. Tissue slices  $\sim 15$ – $20$   $\mu\text{m}$  thick were cut using a Vibratome (Oxford Instruments) and some of the slices were incubated overnight in 0.1 M cacodylate buffer containing 150  $\mu\text{M}$  filipin (given by Dr J. Grady). This procedure ensures a good penetration of filipin throughout the tissue sample. Other slices were stored in buffer alone. For freeze-fracture, the slices were cryoprotected in 30% buffered glycerol for 1 h and then coated with polyvinylalcohol<sup>10</sup>. They were quenched in Freon 22, fractured

at  $-115^\circ\text{C}$  and shadowed in a Balzers 301 freeze-etching device<sup>11</sup>. Replicas were cleaned by sequential treatment with sodium hypochlorite and chloroform-methanol (2:1) before being rinsed in distilled water and recovered on copper grids. They were examined in a Philips EM 300 electron microscope. For thin sectioning, vibratome kidney slices were post-fixed for 2 h in OsO<sub>4</sub>, dehydrated in ethanol and embedded in resin (Epon 812).

Other experiments were performed on homozygous Brattleboro rats with hereditary diabetes insipidus<sup>9</sup> (given by Professor J.-J. Dreyfuss). Three rats were used as untreated controls and three were given daily subcutaneous injections of 1 U of vasopressin (Pitressin tannate in oil; Parke-Davis) for 4 consecutive days. Urine osmolalities were measured immediately before death. Rats were killed by decapitation and the papillae fixed by immersion in 4% glutaraldehyde/0.1 M sodium phosphate buffer. Vibratome slices of the inner medulla were incubated overnight in the presence or absence of filipin



**Table 1** Comparison of the number of particle clusters and IMP density of clusters in control and filipin-treated collecting ducts

|                 | No. of clusters $\mu\text{m}^{-2}$<br>apical membrane | IMP density $\mu\text{m}^{-2}$<br>of clusters |
|-----------------|-------------------------------------------------------|-----------------------------------------------|
| No filipin      | $1.20 \pm 0.10$<br>( $n = 15$ )                       | $1,732 \pm 43$<br>( $n = 20$ )                |
| Filipin-treated | $1.60 \pm 0.12$<br>( $n = 28$ )                       | $1,756 \pm 39$<br>( $n = 20$ )                |

The numbers of clusters per  $\mu\text{m}^2$  of apical membrane were obtained from both P- and E-fracture faces ( $n$  = number of apical membranes quantified). IMP density was counted only on E-faces ( $n$  = number of aggregates examined). In both cases, results are expressed as mean  $\pm$  s.e.m.

and were processed for freeze-fracture and thin-section electron microscopy as described above.

As previously described<sup>2,3</sup>, clusters of intramembrane particles were found on freeze-fractured luminal plasma membranes of collecting duct principal cells after 24 h of water deprivation (Fig. 1a, c). The clusters were visible on both the P- and E-faces of the membrane, but were more conspicuous on the E-face: they contain on average  $\sim 1,700$  IMPs  $\mu\text{m}^{-2}$  compared to only  $350 \mu\text{m}^{-2}$  for the rest of the apical membrane E-face. The number of clusters varied from cell to cell, with an average of  $1.2 \mu\text{m}^{-2}$  (Table 1).

In filipin-treated material, filipin-sterol complexes appear as pits or protuberances  $\sim 25$  nm in diameter on freeze-fractured membrane faces<sup>6</sup>. Thus, these structures can be easily distinguished from the much smaller intramembrane particles. On luminal membranes of collecting duct principal cells, much of the membrane was crowded with filipin-sterol complexes at a density of  $\sim 450 \mu\text{m}^{-2}$ , but patches of membrane corresponding in shape and size to the particle clusters were completely devoid of complexes (Fig. 1b, d). By comparing the IMP density (E-face) of these patches in filipin-treated and untreated material, as well as their frequency in the apical membrane in the two conditions, it was clear that the filipin-free membrane patches corresponded to the particle clusters seen in untreated material as the measured parameters were similar in the presence or absence of filipin (Table 1).

In addition, however, some smaller, circular depressions on the E-face and bumps on the P-face were also partially devoid of filipin-sterol complexes. In material not exposed to filipin, small bumps and depressions can also be seen; these structures correspond to the stubby microvilli present on the apical membranes of these cells.

In thin sections of filipin-treated material, the trilaminar structure of much of the luminal plasma membrane was disrupted by the action of the antibiotic. However, some regions were spared, as in freeze-fractured material; these regions had a cytoplasmic bristle coat and resembled endocytotic coated pits previously described in many other systems (Fig. 1f). These coated membrane areas sometimes appeared flat, but often appeared as slight depressions in the membrane or as invaginated omega figures. Similar coated membrane segments were also found in collecting ducts not exposed to filipin (Fig. 1e) as previously noticed by Bulger and Trump<sup>12</sup>, but in this case the trilaminar structure of all of the apical plasma membrane was retained. By comparing data from freeze-fracture and thin sectioning, these coated membrane regions could be identified as representing the particle clusters seen in freeze-fracture, as both were selectively devoid of filipin-sterol complexes.

The presence of a distinct cytoplasmic bristle coat, a different number of IMPs from the rest of the plasma membrane and an absence of filipin-sterol complexes, are all features characteristic of endocytotic coated pits<sup>7,8,13</sup>, therefore we consider the specialized vasopressin-induced membrane domains on collecting duct principal cells to be coated pits. This was verified by quantitative evaluation of coated pits in Brattleboro rats; the number of pits seen on apical plasma membranes in thin

**Table 2** Quantification of IMP clusters and coated pits in collecting ducts of vasopressin-treated and control Brattleboro rats

| Treatment     | No. of IMP clusters<br>100 $\mu\text{m}^{-2}$<br>membrane area | No. of coated pits<br>100 $\mu\text{m}^{-1}$<br>membrane length |
|---------------|----------------------------------------------------------------|-----------------------------------------------------------------|
| + Vasopressin | $109.5 \pm 9.9$<br>( $n = 51$ )                                | $9.74 \pm 1.49$<br>( $n = 205$ )                                |
| Control       | 0<br>( $n = 36$ )                                              | $0.82 \pm 0.09$<br>( $n = 171$ )                                |

Data were obtained from three control and three vasopressin injected Brattleboro rats.  $n$ , number of cells examined. Results are expressed as mean  $\pm$  s.e.m. IMP clusters were counted on freeze-fracture replicas of apical membranes from collecting duct principal cells (P- and E-faces). Coated pits were counted in thin sections of collecting ducts.

sections was greatly increased after vasopressin administration to three animals (Table 2). This increase occurred in parallel with the previously reported<sup>2,3</sup> increase in IMP clusters in these conditions (Table 2). However, a few coated pits were present in control Brattleboro rats, whereas particle clusters were virtually absent, suggesting that a small percentage of coated pits are not associated with the characteristic particle aggregates.

Whether the vasopressin-induced particle aggregates in amphibian bladder and epidermis are functionally related to the collecting duct clusters is unknown, but in freeze-fracture they are different, the intramembrane particles being more tightly packed than in the kidney; whether they also correspond to coated membrane segments has not been determined. We have previously reported that filipin-sterol complexes are also absent from vasopressin-induced aggregates in the toad bladder<sup>14</sup> but in such highly particulate membrane domains, steric factors can prevent the formation of 25-nm filipin-sterol complexes. In the kidney, the particle clusters are much less tightly packed and the absence of complexes may reflect either a low cholesterol content or the existence of other as yet undetermined membrane constraints which restrict visible complex formation (for discussion, see ref. 15).

Nevertheless, whatever the reason for the absence of visible filipin-sterol complexes from these particle clusters, this fact has enabled vasopressin-induced membrane specializations to be unambiguously identified in thin sections as coated pits in the collecting duct. Coated pits are, in other systems, involved in receptor-mediated endocytosis<sup>8</sup> and their induction on luminal cell membranes that are in contact with the urine is an intriguing phenomenon, as vasopressin acts via receptors on the basolateral membrane of these cells<sup>16</sup>. How coated pits induced on the luminal membranes of kidney collecting duct principal cells are related to the antidiuretic action of vasopressin now remains to be clarified.

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## Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination

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Recent experiments in the murine system<sup>1,2</sup> have indicated that the passive acquisition by offspring of maternal anti-malarial IgG antibodies while conferring some degree of immunity against a primary infection, paradoxically prevents the generation of acquired immunity through vaccination. Therefore, in view of earlier findings<sup>3</sup> concerning the competitive effects of specific IgM and IgG antibodies, we investigated whether specific monoclonal IgM antibodies could be used to potentiate the response to a blood-stage murine malaria vaccine. We now report that small amounts of purified monoclonal anti-parasite IgM can specifically potentiate both priming and memory cell generation in response to vaccination as evidenced by survival after infection, and that the magnitude of this effect is greater than that found with a more conventional nonspecific adjuvant (*Bordetella pertussis*). Additionally, in offspring of immune mothers, where vaccination is ineffective for up to 8 weeks due to the presence of maternal IgG<sup>1,2</sup>, we have found that IgM when administered with the vaccine can completely overcome this inhibition by its adjuvant effect.

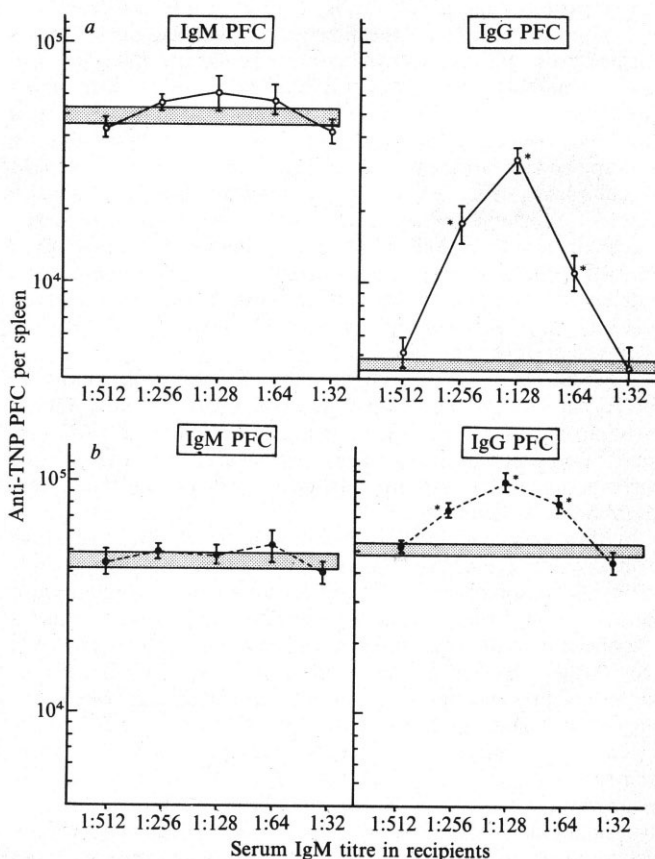
Spleen cells from an immune *Plasmodium yoelii*-infected BALB/c mouse were fused with P3-X63-Ag8.653 myeloma cells<sup>4</sup> in the presence of polyethylene glycol, as described previously<sup>5</sup>. Of 98 cultures tested by immunofluorescence<sup>6</sup> 22 were positive, with 2 producing parasite-specific IgM. These lines (APY.1 and APY.2) were subsequently cloned and the antibody purified from the ascitic fluid. As APY.1 and APY.2 gave virtually identical results, only the findings for APY.1 will be reported here.

The origin of our lethal *P. yoelii* parasite and the preparation of a crude formalin-fixed blood-stage malaria vaccine have been described previously<sup>7</sup>. Normal (BALB/c × C57BL/6)F<sub>1</sub> mice when given a single intravenous (i.v.) injection of  $5 \times 10^7$  formalin-fixed *P. yoelii* combined with *B. pertussis* develop a brief parasitaemia followed by complete sterile immunity. However, as previously reported<sup>2,3</sup>, 4-week-old offspring of immune mothers fail to be protected by a similar vaccination regime due to the transfer of maternal IgG. To determine if monoclonal IgM (APY.1) could overcome this inhibition and also replace the requirement for *B. pertussis* as an adjuvant, the (BALB/c × C57BL/6)F<sub>1</sub> progeny of immune or normal BALB/c females were weaned at 3 weeks and vaccinated at 4 weeks of age using *B. pertussis* or APY.1 as the adjuvant. Table 1 summarizes the results of two experiments involving immunization and challenge of 190 offspring. As assessed by survival, offspring of immune mothers were completely unprotected by vaccination when *B. pertussis* was used; however when APY.1 was administered at an optimal dose (1:128) all mice were completely protected, developed high titres of specific IgG antibody and had only a slight delay in recovery; at no time was any protection observed when IgM was administered in the absence of antigen or when the vaccine was administered with an irrelevant IgM monoclonal antibody. All mice born to normal mothers were successfully vaccinated whether *B. pertussis* or IgM was used as the adjuvant; at the lowest doses of IgM (1:64 and 1:32) some delay in recovery was noted. Contrary to the action of *B. pertussis*, the enhancing action of the IgM was specific, as the responses to an unrelated antigen, sheep erythrocytes, were unaffected (data not shown).

To determine if the T helper cell (T<sub>H</sub>) response, known to be suppressed in offspring of immune mothers<sup>2</sup>, was the principal target of this IgM-mediated enhancement, we used the approach of coupling a standard hapten trinitrophenol (TNP) to the free parasite and measuring the anti-TNP plaque-forming cell (PFC) response; in this way recognition of the parasite as a carrier can be quantitated<sup>6</sup>. As Fig. 1 shows, maximum enhancement of memory T<sub>H</sub>-cell generation occurs when an optimal amount of APY.1 (equivalent to a titre of 1:128 in the recipients) is injected before vaccination; responses in the suppressed offspring of immune mothers (Fig. 1a) are increased ninefold to 60% of a normal response, while those in offspring of non-immune mothers (Fig. 1b) are twofold greater. It is interesting that the above enhancement is restricted to those T<sub>H</sub> cells involved in the IgG component of the PFC response.

As it has been suggested that this phenomenon is due to increased concentration of antigen in the spleen<sup>8</sup>, the effect of monoclonal IgM on antigen localization in the spleens of normal (BALB/c × C57BL/6)F<sub>1</sub> mice was measured 24 h after injection of radiolabelled vaccine. As Table 2 shows, there was no correlation between maximal spleen localization and optimal enhancement.

To determine if the IgM must be present early in the immune response to mediate this enhancement, groups of 4-week-old (BALB/c × C57BL/6)F<sub>1</sub> mice from immune or normal mothers were injected with optimal amounts of APY.1 (see Fig. 1) at various times relative to vaccination and their T<sub>H</sub>-cell responses measured 2 weeks later. As Fig. 2 shows, the IgM must be



**Fig. 1** Enhancement of memory T<sub>H</sub>-cell responses by IgM. Four-week-old offspring of immune (○—○) or normal (●—●) mothers were injected i.v. with graded amounts of APY.1 2 h before vaccination with  $5 \times 10^7$  FFPY. Two weeks later they were challenged with  $10^5$  FFPY. Two weeks later they were challenged with  $10^5$  haptenated parasites (TNP-PY) and the splenic TNP-specific PFC (see ref. 22) measured 4 days later. The values shown are the geometric means  $\pm 1$  s.e. for groups of at least 10 mice. The shaded areas denote the responses ( $\pm 1$  s.e.) in the respective groups when *B. pertussis* replaced IgM as the vaccine adjuvant. The serum IgM titres in the recipients were measured just before vaccination. \*  $P < 0.001$  using Student's *t*-test.

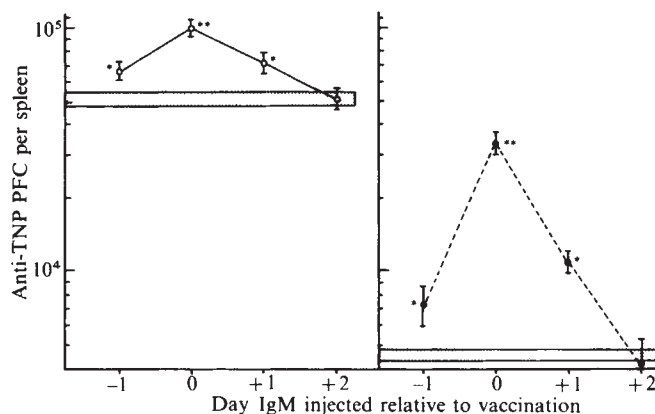
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**Table 1** Protection by vaccination against *P. yoelii* using IgM as an adjuvant

| Mother  | Vaccine | Adjuvant                            | Responses to infection |                      |                                 |          |
|---------|---------|-------------------------------------|------------------------|----------------------|---------------------------------|----------|
|         |         |                                     | No. recovered/total    | Mean day of recovery | Serum IFA titre (day 10)<br>IgM | IgG      |
| Immune  | +       | 10 <sup>8</sup> <i>B. pertussis</i> | 0/20                   | —                    | 1:1,000                         | 1:2,000  |
| Immune  | +       | APY IgM (1:256)                     | 8/12                   | 18.8                 | 1:1,000                         | 1:8,000  |
| Immune  | +       | APY IgM (1:128)                     | 12/12                  | 11.2                 | 1:1,000                         | 1:32,000 |
| Immune  | +       | APY IgM (1:64)                      | 3/12                   | 21.5                 | 1:1,000                         | 1:4,000  |
| Immune  | +       | SRBC IgM (1:128)                    | 0/6                    | —                    | 1:1,000                         | 1:1,000  |
| Immune  | —       | APY IgM (1:128)                     | 0/12                   | —                    | 1:256                           | 1:256    |
| Control | +       | 10 <sup>8</sup> <i>B. pertussis</i> | 20/20                  | 7.8                  | 1:1,000                         | 1:32,000 |
| Control | +       | APY IgM (1:256)                     | 12/12                  | 9.2                  | 1:1,000                         | 1:32,000 |
| Control | +       | APY IgM (1:128)                     | 12/12                  | 8.8                  | 1:1,000                         | 1:64,000 |
| Control | +       | APY IgM (1:64)                      | 12/12                  | 9.0                  | 1:1,000                         | 1:32,000 |
| Control | +       | SRBC IgM (1:128)                    | 6/6                    | 12.5                 | 1:1,000                         | 1:16,000 |
| Control | +       | —                                   | 20/20                  | 12.2                 | 1:1,000                         | 1:16,000 |
| Control | —       | APY IgM (1:128)                     | 0/12                   | —                    | 1:256                           | 1:512    |

Female BALB/c mice were vaccinated with  $5 \times 10^7$  formalin-fixed *P. yoelii* (FFPY), infected 2 weeks later and when their serum contained high titres of anti-malarial antibody (see ref. 2) they were mated to normal C57BL/6 males. Control matings were with BALB/c females immunized with a placebo. The 4-week-old offspring of immune or control mothers were injected with graded amounts of parasite-specific (APY) or an irrelevant monoclonal IgM specific for sheep erythrocytes (SRBC) 2 h before i.v. vaccination with  $5 \times 10^7$  FFPY (the figures in parentheses denote the passive IgM titre in the recipients at vaccination); alternatively the vaccine was administered in *B. pertussis*. Two weeks later, all animals were challenged i.v. with  $10^4$  live *P. yoelii*. Immunofluorescent antibody (IFA) titres were measured on *P. yoelii* dried films (see ref. 7).



**Fig. 2** The afferent action of the enhancement by IgM. Four-week-old offspring of immune (●—●) or normal (○—○) mothers were injected with an optimal amount of IgM (see g. 1) at various times relative to vaccination with  $5 \times 10^7$  FFPY. Two weeks later the mice were challenged with  $10^5$  TNP-PY and the splenic TNP-specific IgG PFC measured 4 days later. The values shown are the geometric means  $\pm$  s.e. for groups of at least eight mice. The shaded areas denote the responses in the respective groups when *B. pertussis* replaced IgM as the adjuvant.

\*  $P < 0.001$  using Student's *t*-test; \*\*  $P < 0.0001$ .

**Table 2** Effect of monoclonal IgM on vaccine localization in the spleen

| Titre of IgM in recipients | % Localization in spleen | % Increase in splenic localization | Significance |
|----------------------------|--------------------------|------------------------------------|--------------|
| 1:512                      | $3.34 \pm 0.39$          | 50.5                               | $P < 0.001$  |
| 1:256                      | $2.92 \pm 0.09$          | 31.5                               | $P < 0.001$  |
| 1:128                      | $3.05 \pm 0.21$          | 37.4                               | $P < 0.001$  |
| 1:64                       | $2.71 \pm 0.24$          | 22.1                               | $P < 0.01$   |
| 1:32                       | $2.82 \pm 0.24$          | 27.0                               | $P < 0.01$   |
| —                          | $2.22 \pm 0.27$          | 0                                  | —            |

Normal (BALB/c  $\times$  C57BL/6)F<sub>1</sub> mice were injected with graded amounts of APY.1 IgM 2 h before i.v. inoculation with  $5 \times 10^7$  FFPY radiolabelled, using the iodogen method, with <sup>125</sup>I. Twenty-four hours later the spleens were removed, counted in an LKB Wallace gamma counter and the percentage localization calculated. The values shown represent the means  $\pm$  s.d. for groups of at least six mice. The specific serum IgM titre in the recipients was measured just before vaccination. The significance was calculated using Student's *t*-test.

present just before or during the first few hours after priming to mediate its potentiating effect on IgG memory.

Although it is well documented that IgM antibodies will specifically enhance an immune response<sup>1,8-10</sup>, very little is known about the underlying mechanisms involved. From the results above and our similar preliminary findings with specific polyclonal IgM (ref. 11), it is clear that the protection is not due simply to increased localization of the vaccine in the spleen (see Table 2) or to an antigen-independent mechanism (see Table 1), such as modulation of the idiotype network<sup>12</sup>. The observed enhancement of the immune response to vaccination is specific, antigen-dependent (see Table 1 and Fig. 1) and, in view of the optimal titre required, seems more likely to result from the binding of an optimal amount of IgM to the antigen. Furthermore, the kinetics (Fig. 2) and the magnitude (Fig. 1) of the enhanced response relative to that induced by either antigen or IgM alone would suggest some form of synergy in the induction of the response.

Antigen-reactive B cells are optimally stimulated by signals from two distinct T<sub>H</sub> cells, one specific for antigen (T<sub>H</sub>1)<sup>13</sup> and the other specific for both carrier and idiotype (T<sub>H</sub>2)<sup>14</sup>. As the T<sub>H</sub>2 cell is known to amplify an immune response and has also been implicated in isotype switching<sup>15</sup> and memory B-cell triggering<sup>16</sup>, it is conceivable that the magnitude of the observed enhancement at the correct IgM: antigen ratio could result from optimal priming of the T<sub>H</sub>1 and T<sub>H</sub>2 cells via their Fc receptors for aggregated IgM<sup>17</sup>; enhancement at the T<sub>H</sub>2 cell level would also explain the restriction of the effect to the IgG component of the memory responses (see Table 1 and Fig. 1) and also its carrier specificity.

A major drawback in the development of malaria vaccine suitable for use in a human trial is the unavailability of a suitable adjuvant; experimental merozoite vaccines in the widely-used simian system require Freund's complete adjuvant which is not acceptable for use in man<sup>18</sup>. Furthermore, if a malaria vaccine is to be of maximum benefit it must protect neonates born to mothers in endemic areas where the high titres of maternal IgG (ref. 19) may impair its effectiveness. In view of these requirements and our findings showing the ability of an IgM adjuvant specifically and physiologically to overcome maternal inhibition of a blood-stage malaria vaccine, it would be of interest to determine if merozoite vaccination could be similarly enhanced in the simian system, using specific IgM as an adjuvant. Specific IgM may also be of use in overcoming a similar maternal inhibition of measles<sup>20</sup> and poliomyelitis<sup>21</sup> vaccination in tropical areas.

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## Ethanol-induced chromosomal abnormalities at conception

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**Preliminary findings have indicated that mouse eggs exposed briefly *in vivo* or *in vitro* to a dilute solution of ethanol activate parthenogenetically<sup>1,2</sup>. Cytogenetic analysis of the first-cleavage chromosomes of haploid parthenogenetic embryos indicated that up to 20% of this population were aneuploid as a result of non-disjunction<sup>2</sup>. Anaesthetics also can induce parthenogenesis of rodent eggs<sup>3–5</sup>, and in studies using anaesthetics<sup>6</sup>, colchicine<sup>3,7–9</sup> and colcemid<sup>10</sup>, abnormal chromosome segregation and heteroploidy of rodent embryos have been observed. I now report that when recently mated female mice are given a dilute solution of ethanol by mouth, non-disjunction can be induced in the female-derived, but apparently not in the male-derived, chromosome set of fertilized eggs. Taken together, these findings suggest that ethanol consumption (as well as exposure to other 'spindle-acting' agents<sup>11,12</sup>) at the time of conception may be the cause of certain types of chromosomal defects commonly observed in human spontaneous abortions.**

The initial studies were designed to investigate the chromosome constitution of haploid embryos induced to develop parthenogenetically following ethanol administration by various routes to unmated female mice. The same high levels of activation can be achieved either using a dilute solution of ethanol administered via the intraperitoneal (i.p.) route<sup>1</sup> or following oral ethanol administration (M.H.K., unpublished). Maximum rates of activation (50–60%) can be achieved (M.H.K., unpublished) when mice are injected i.p. with 0.3–0.4 ml of a 25% solution of ethanol in phosphate-buffered saline at 17 h after human chorionic gonadotropin (HCG) injection for superovulation. Detailed examination of the first-cleavage chromosomes from embryos in this group revealed that 7.5% of the haploid mitoses were aneuploid. Out of a total of 133 preparations in which unequivocal counts could be made (63% of preparations examined), 123 metaphases had a normal chromosome complement, 5 contained 19 and 5 contained 21 chromosomes.

Activation could also be induced when lightly anaesthetized mice (ether inhalation) were given 1 ml of a 10% solution of ethanol in distilled water through a gastric tube at a similar time after induced ovulation. Out of a total of 42 preparations in which unequivocal counts could be made (100% of preparations examined), 40 had a normal chromosome complement, one group had 19 and another had 21 chromosomes present.

Note that parthenogenetic mouse embryos, even with an apparently normal haploid or diploid chromosome constitution, generally fail to develop beyond the early post-implantation period<sup>13</sup>.

These preliminary results encouraged an investigation of the effect of ethanol exposure shortly after conception. Recently mated mice were given 1 ml of either a 10%, 12.5% or 15% solution of ethanol in distilled water by mouth at 13.5 h after the HCG injection for superovulation (about 1.5–2.5 h after the predicted time of ovulation). Serum levels of ethanol of about 150–200 mg ml<sup>-1</sup> are achieved within minutes of its administration<sup>14</sup>, and maximum levels would be expected in the oviduct shortly afterwards, during the time that eggs are completing the second meiotic division—shortly after the 'activating' stimulus of fertilization. Table 1 shows that in 15% of the eggs, one of the pronuclear sets contained 20 and the other 19 or 21 chromosomes. In this series, unequivocal chromosome counts could be made in 65.3% of the preparations studied.

However, the absence of an appropriate 'marker' chromosome in either the male- or female-derived pronuclear groups precluded the origin of the aneuploid sets being established with any degree of certainty.

Thus, in a second series of experiments, (C57BL × CBA)F<sub>1</sub> hybrid females were mated with males that were homozygous for the T6 translocation in which a reciprocal exchange occurs between segments of chromosomes 14 and 15, with the resultant production of a large and a small translocation product. The latter is easily recognizable as it is considerably smaller than the smallest intact autosome, and has been termed the T6 marker chromosome or T6M<sup>15–17</sup>. In such matings, the sperm-derived pronuclear set would be expected to contain 20 chromosomes, including one T6M chromosome. Pronuclear groups could therefore be scored as male- or female-derived depending on the presence or absence, respectively, of the T6M chromosome (see Fig. 1). I found that all the male-derived pronuclear chromosome groups (containing the T6M chromosome) had 20 chromosomes present. In the female-derived sets, however, while many contained groups with the normal complement of 20 chromosomes, almost one-fifth of the eggs studied had female-derived pronuclear groups with either 19 or 21 chromosomes present (Table 1).

In control females exposed to similar low levels of anaesthesia in which an equal volume of distilled water was introduced via a gastric tube, no aneuploidy was observed (Table 1), nor was aneuploidy observed in parthenogenetic studies in which eggs were activated *in vitro* in the absence of ethanol<sup>2</sup>. Similarly, when unfertilized eggs isolated from mated females were activated *in vitro* by handling alone<sup>18</sup> 6–7 h after the females had been given ethanol by mouth, no evidence of aneuploidy was observed in the haploid population examined (Table 1).

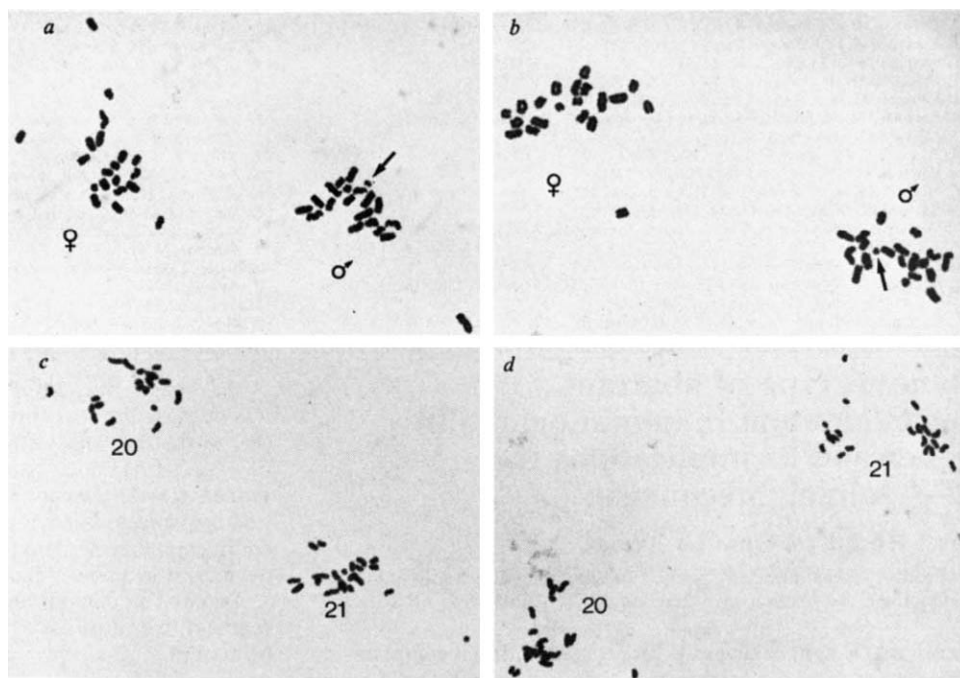
The studies described here clearly demonstrate that *in vivo* exposure of parthenogenetically activated and recently fertilized mouse eggs to ethanol while they are completing the second meiotic division may induce non-disjunction. In the fertilized eggs, no evidence of non-disjunction involving the male pronuclear chromosomes was observed following exposure of embryos to ethanol at this time.

Many laboratory animal studies have unequivocally demonstrated that brief exposure to either ethanol<sup>19–22</sup> or its primary metabolite acetaldehyde<sup>23,24</sup> may be teratogenic during the early post-implantation period. The effects of maternal alcohol consumption on human fetal development—ranging from low birth weight infants to the birth of severely mentally retarded and congenitally abnormal children—are also well recognized<sup>25–28</sup>. In these studies it has been assumed that ethanol produces its most harmful effects during early embryogenesis<sup>25,29</sup>. The present study, however, indicates that ethanol may be even more harmful than generally appreciated, because of the vulnerability of the conceptus during the first few hours following fertilization.

While the absolute extent of the damage induced during



**Fig. 1** Air-dried preparations of male and female pronuclear chromosome groups analysed at the first cleavage mitosis. After overnight incubation of eggs in culture medium containing colcemid, two discrete sets of chromosomes can be observed. The preparations were stained with Giemsa. *a, b*, (C57BL  $\times$  CBA) $F_1$  hybrid females were mated to males that were homozygous for the T6 translocation. The male-derived pronuclear chromosome groups containing the T6 marker chromosome (arrowed) are on the right. *c, d*, Pronuclear chromosome groups from a study in which  $F_1$  hybrid females were mated to males of similar genotype. In both cases, the group on the left contains 20 chromosomes whereas the group on the right contains 21 chromosomes. Indirect evidence from parthenogenetic studies and fertilized matings in which the males carried appropriate marker chromosomes (see *a* and *b*, above) indicates that in these examples, the groups containing 21 chromosomes were of female pronuclear origin.



morphogenesis would be almost impossible to quantify due to the effect of tissue regeneration and compensatory growth, no amount of compensatory growth would be capable of restoring the normal chromosome complement in an aneuploid individual, and most of these fetuses would either be aborted spontaneously or, with very few exceptions, die shortly after birth<sup>30</sup>. Indeed, two recent epidemiological studies have shown that a significant relationship exists between the level of maternal alcohol intake during early pregnancy and the risk of spontaneous abortion<sup>31,32</sup>, and there seems little reason to believe that the pattern of alcohol consumption during early pregnancy is likely to be radically different from that occurring before and shortly after conception. The reason for the higher incidence of spontaneous abortions in the alcohol-consuming groups was not established, but the present findings suggest that a cytogenetic explanation might be applicable in some cases.

While caution should be exercised in extrapolating directly from the present findings, as there are likely to be certain species differences in ethanol susceptibility, the results draw attention to the potential danger to the conceptus of a single episode of heavy drinking by the mother at about the time of conception. They also indicate that any agent which acts on the spindle apparatus, and could therefore influence meiotic chromosome segregation, should be considered a possible causative agent in those cases of spontaneous abortion in man in which a non-mosaic type of chromosomal defect (for example, trisomy, monosomy and certain polyploids) is observed. These groups probably account for at least one-half of all spontaneous abortions in man<sup>30</sup>.

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**Table 1** The chromosome constitution of male and female pronuclei following intragastric ethanol administration

| Group | % Ethanol*                | Total no. eggs examined | % Scorable preparations | 18:20 | 19:20       | 20:20       | 21:20       | % Aneuploid |
|-------|---------------------------|-------------------------|-------------------------|-------|-------------|-------------|-------------|-------------|
| 1     | 10                        | 93                      | 63.4                    | 1     | 7           | 50          | 1           | 15.3        |
| 2     | 12.5                      | 58                      | 63.8                    | 1     | 3           | 30          | 3           | 18.9        |
| 3     | 15                        | 22                      | 77.3                    |       |             | 16          | 1           | 5.9         |
| 4     | 12.5                      | 79                      | 84.8                    |       | 19:20 (T6M) | 20:20 (T6M) | 21:20 (T6M) | 17.9        |
| 5     | Distilled water (control) | 18                      | 66.7                    |       | 7           | 55          | 5           | 0           |
|       |                           |                         |                         |       |             | 12          |             |             |
| 6     | Haploids†                 | 17                      | 100                     |       | 19          | 20          | 21          | 0           |
|       |                           |                         |                         |       |             | 17          |             |             |

\* All females received 1 ml of a dilute solution of ethanol in distilled water, or distilled water only (group 5).

† Activation induced *in vitro* 6–7 h after intragastric administration of 12.5% ethanol.

6- to 8-week old (C57BL  $\times$  CBA) $F_1$  hybrid females were injected with 5 IU pregnant mare's serum gonadotropin followed 48 h later by 5 IU HCG to synchronize ovulation, and were mated to  $F_1$  hybrid (groups 1–3) or CBA-T6T6 (groups 4–6) males. Females were checked for vaginal plugs early the next morning, and at 13.5 h after the HCG injection for superovulation those that had mated were lightly anaesthetized with ether so that the test solutions could be introduced via a fine tube passed into the stomach. Most females recovered from the anaesthetic within a few minutes of the intragastric injection. The females were autopsied 6–7 h later, and the eggs examined for the presence of pronuclei. The fertilized eggs were transferred to medium containing colcemid, and air-dried preparations were made the next morning. The unfertilized eggs were examined 5–7 h later for evidence of parthenogenetic activation (the development of a single pronucleus following second polar body extrusion), and the activated eggs then transferred to a separate drop of medium containing colcemid. These eggs were also examined the next morning, and their chromosome constitution determined. Following incubation in colcemid, the chromosomes of the male and female pronuclei in the fertilized eggs remain as two distinct entities, and the chromosomal constitution of each can then be determined. Only in groups 4 and 5 did the presence of the T6 marker chromosome (T6M) enable the male-derived pronuclear set of chromosomes to be established unequivocally.

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## A novel type of aberrant recombination in immunoglobulin genes and its implications for V-J joining mechanism

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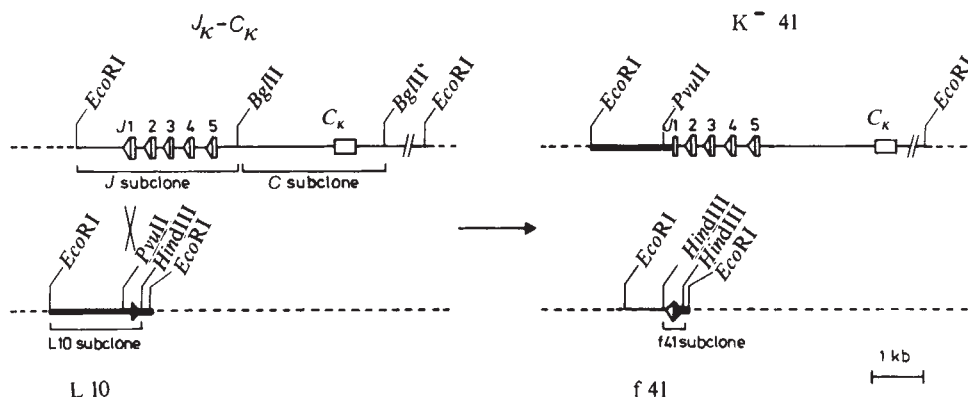
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**Functional  $\kappa$  light chain genes are formed during B-lymphocyte differentiation by the joining of initially separate V and J gene segments<sup>1,2</sup>. It has been suggested that the intervening DNA is deleted<sup>1,2</sup>, however the recent reports of what appear to be the reciprocal products of V and J recombination (back-to-back conserved V and J flanking sequences, called f-fragments) in DNA from mature lymphocytes make a simple deletion model unlikely<sup>3–7</sup>. An alternative scheme involving unequal sister chromatid exchange<sup>5,6</sup> has been proposed, supported by the evidence that the f-fragments seem to have segregated from the chromosome carrying the reciprocal complete  $\kappa$  light chain gene (this and other schemes are briefly reviewed in ref. 8). We report here the analysis of a mouse myeloma (MOPC 41), in which a productive ( $\kappa^+$ )<sup>9</sup> and a non-productive ( $\kappa^-$ )<sup>6,10</sup> rearrangement has occurred, which may help to clarify the mechanism of V-J joining. The aberrant rearrangement has led to the joining of a  $J_1$  gene segment to a sequence unrelated to any V gene (L10), and which in the germ line is flanked by a sequence resembling a V region recombination signal sequence. In this case no segregation of the reciprocal recombination products ( $\kappa^-41$  and f41), which is a required step in sister chromatid exchange models, has taken place. An inversion model provides the simplest explanation of this J rearrangement.**

\* J.H. died on 27 August 1982.

**Fig. 1** The  $\kappa^-41$  rearrangement. In MOPC 41 a segment of non-V gene DNA (L10) has been recombined with  $J_1$  to generate the  $\kappa^-41$  sequence and the reciprocal flank recombination product, f41. The heptanucleotide signal sequences<sup>1,2</sup> are indicated as triangles (triangles and J boxes not to scale), L10 sequences as thick lines.

**Methods:** The 1.9-kb *EcoRI* fragment L10 was cloned in  $\lambda$ gtWES, from a mouse liver DNA digest after enrichment by RPC5 chromatography (as in ref. 3) using the f41 subclone<sup>6</sup> as probe. Two independent clones were analysed (Fig. 2). Blot hybridizations of restriction nuclease digests of liver DNA in stringent conditions with the f41 subclone revealed only one strongly hybridizing band (1.9 kb with *EcoRI*, and 6.1 kb with *BamHI*), and the weakly hybridizing germ-line  $J_\kappa$  fragments (data not shown). A 1.88-kb *EcoRI*–*HindIII* fragment of L10 was subcloned in pBR322. The other subclones have been published previously<sup>3,6</sup>. Analogously, the 15-kb *EcoRI* fragment  $\kappa^-41$  was cloned in Charon 4A from RPC5 chromatography fractions of a MOPC 41 DNA digest<sup>6</sup>, using the L10,  $J_\kappa$  and  $C_\kappa$  subclones as probes.  $\kappa^-41$  clones<sup>9</sup> were also obtained, but among six  $J_\kappa$ -positive clones no germ-line  $J_\kappa$ – $C_\kappa$  clone was found (as expected from previous hybridization data<sup>6,10</sup>, but different from another report<sup>9</sup>).



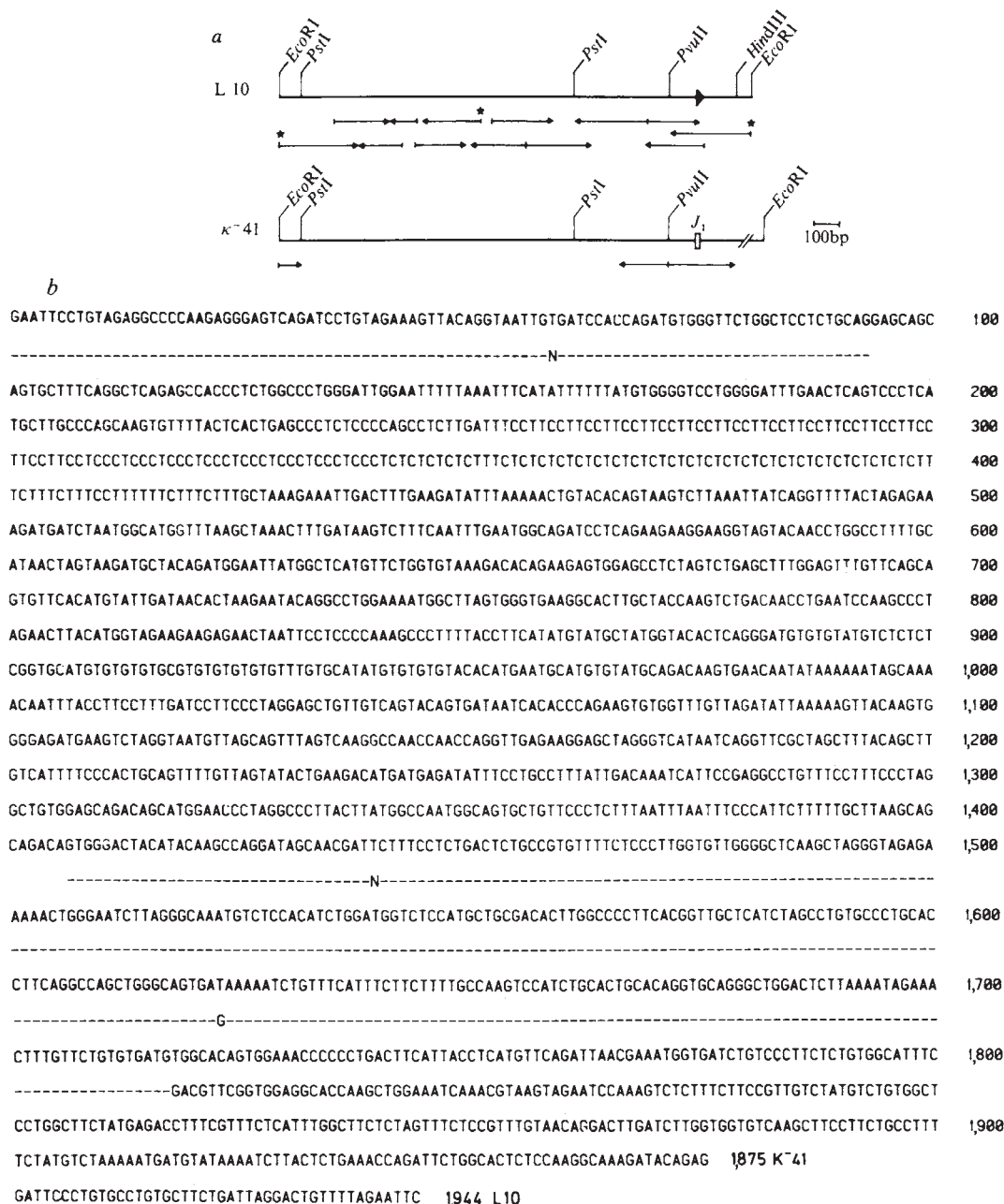
One reason why MOPC 41 was chosen for further analysis was because the structure of f41 had been found to differ from that of the previously studied f-fragments fT and f173 (refs 3, 6). All f-fragments analysed so far contain a  $J_1$  flanking sequence, with the canonical heptanucleotide and nonanucleotide sequences; but while fT and f173 also have a complete, complementary V flanking sequence, f41 has only the heptanucleotide sequence, and lacks a nonanucleotide sequence<sup>6</sup>.

As a first step in our analysis we isolated the germ-line DNA segment that must have been joined to  $J_1$  in the process of f41 formation. This was accomplished using the previously described f41 subclone<sup>6</sup> as hybridization probe (Fig. 1). The isolated fragment (L10) was sequenced (Fig. 2). A subclone prepared from L10 was used in the second step of our analysis to probe for homologous sequences in MOPC 41 DNA digests. This allowed us to clone the  $\kappa^-41$  fragment, which was subsequently partly sequenced (Fig. 2).

From the heptanucleotide CACAGTG at positions 1,721–1,727 to the end of the known sequence at position 1,944 (Fig. 2), the sequence of L10 was found to be identical to that of f41 (ref. 6, not shown here). Thus, L10 is almost certainly the germ-line equivalent of this part of f41. The surprising feature revealed by the sequence of L10 is the absence of a V gene segment at the 5' side of the heptanucleotide sequence. f41 thus seems to have been formed by the recombination of  $J_1$  with the non-immunoglobulin gene segment L10.  $\kappa^-41$  must have been formed during the same recombination, as the entire 540 base pairs (bp) that were sequenced are identical to the corresponding sequences of L10 and  $J_1$  (refs 1, 2) except for one difference (position 1,623; Fig. 2) which may be due to a somatic mutation. This is the basis of the scheme in Fig. 1 which shows  $\kappa^-41$  and f41 as reciprocal recombination products.

No pronounced homologies were detected between the L10 sequence and any known DNA or protein sequences (Fig. 2).

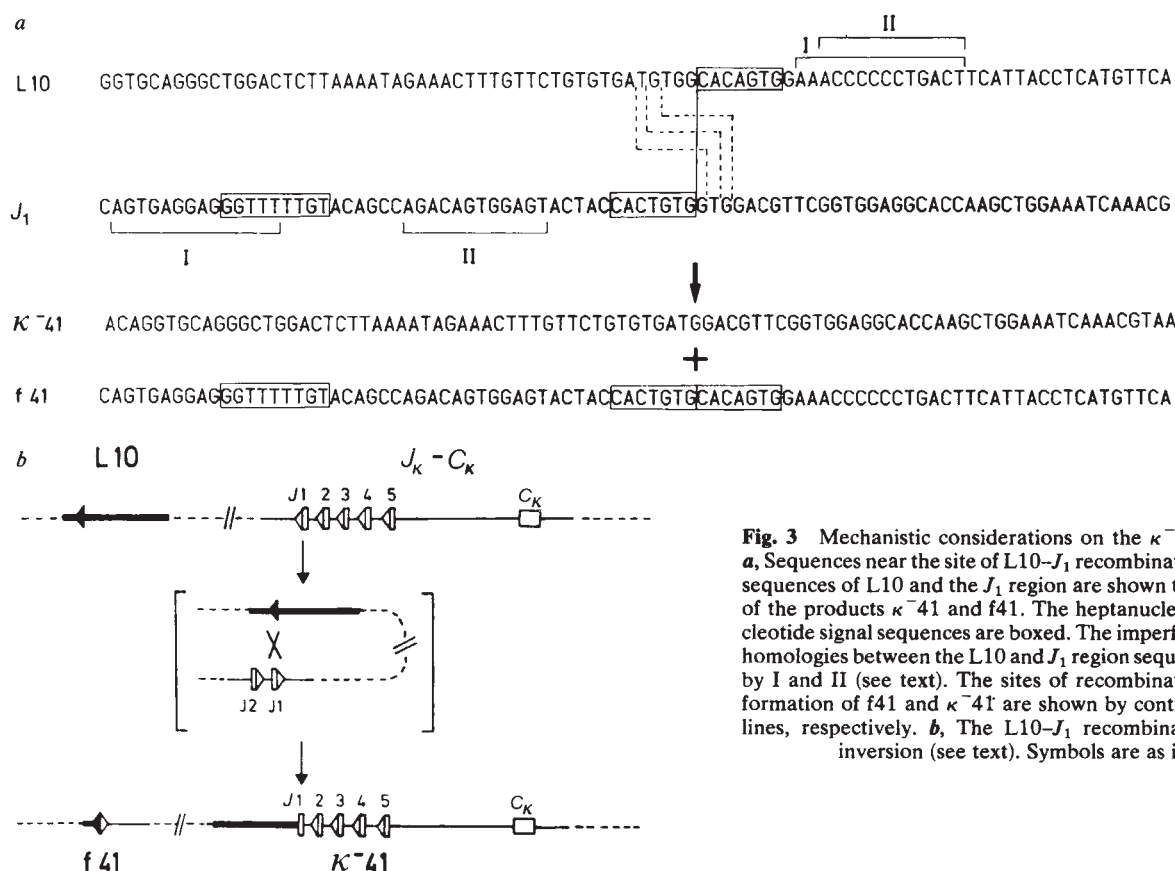




**Fig. 2** **a**, Restriction maps and sequencing strategies for L10 and the rearranged part of  $\kappa^{-41}$ . Sequencing was by the method of Maxam and Gilbert<sup>11</sup>. Fragments of L10 marked by asterisks were derived from the *EcoRI* insert of the *lgtWES* clones (Fig. 1). In addition to the ends a single-strand nick at an *EcoRI* site was strongly labelled and could be used. All other fragments were derived from the L10 subclone (upper line, *DdeI* fragments; bottom line, *Sau3A* fragments). For sequencing of  $\kappa^{-41}$ , the inserts were isolated from the Charon phages. Sequences around the recombination sites were determined in two independent clones each of L10,  $\kappa^{-41}$  and f41 in order to avoid cloning artefacts. **b**, Sequence of fragment L10 (upper lines) and partial sequence of  $\kappa^{-41}$  (lower lines). The heptanucleotide sequence involved in recombination at positions 1,721–1,727. Sequences of  $\kappa^{-41}$  (positions 1–93, 1,407 onwards) are indicated by a broken line where identical to those of L10. Two nucleotides were not read (N at positions 59 and 1,440), and one deviates from L10 (position 1,623). From position 1,718 onwards, the  $\kappa^{-41}$  sequence is identical to that of the *J<sub>K</sub>* region (starting with position 88; ref. 1). The sequence of f41, most of which was published previously<sup>6</sup>, was completed and shown to be identical to the respective part of L10. A computer-aided search for homologies was carried out at Rechenzentrum, Max-Planck-Institut für Biochemie, Martinsried. In a comparison of the L10 sequence (both strands) to the Nucleic Acid Sequence Data Base of Dayhof *et al.* (version 15; 2 April 1982) pronounced homologies were detected only to the repetitive sequences of L10 (using a unitary scoring matrix, and comparing 50-bp segments). Also, comparison of the three translation frames each of both strands of the DNA sequence to the Protein Sequence Data Base of Dayhof *et al.* (version 4; 30 April 1982) did not reveal any pronounced homologies using the mutation data matrix and segments of 25 amino acids.

legend). Blot hybridizations using the L10 subclone (data not shown) have shown that L10 sequences are present in the germ-line context in several  $\kappa$  chain-producing myelomas (MOPC 21A, 41, 173, MPC 11, myeloma T). The observation that both the L10 and  $C_{\kappa}$  sequences are deleted in the  $\lambda$  light chain-producing tumour RPC 20 (data not shown) may indicate a physical linkage between the two DNA segments. No other data are available on the location of L10 within the mouse genome. An interesting structural feature of L10 is the occur-

rence of simple DNA sequences (for example at positions 252–425 and 884–948, Fig. 2). Similar sequences have been found near many genes, and possibly at other locations (see, for example, refs 12–16), and may contribute to the mobility of the neighbouring DNA segments within the genome. The presence of simple sequences probably explains the high background, and the appearance of several bands when blot hybridizations were carried out with the L10 subclone in relaxed conditions.



**Fig. 3** Mechanistic considerations on the  $\kappa^{-41}$  rearrangement. **a**, Sequences near the site of L10- $J_1$  recombination. The germ-line sequences of L10 and the  $J_1$  region are shown together with those of the products  $\kappa^{-41}$  and f41. The heptanucleotide and nonanucleotide signal sequences are boxed. The imperfect inverted repeat homologies between the L10 and  $J_1$  region sequences are indicated by I and II (see text). The sites of recombination leading to the formation of f41 and  $\kappa^{-41}$  are shown by continuous and broken lines, respectively. **b**, The L10- $J_1$  recombination drawn as an inversion (see text). Symbols are as in Fig. 1.

The sequences near the site of L10- $J_1$  recombination deserve particular attention (Fig. 3a). The six nucleotides that are immediately adjacent, but on opposite sides of the site of recombination, (TGT/GGG, GTG/GGT or TGG/GTG) are absent from the recombination products  $\kappa^{-41}$  and f41. A few nucleotides are probably also deleted in the joining of  $J$  and  $V_\kappa$  segments<sup>3,6</sup>. Three different joining sites could have generated the  $\kappa^{-41}$  sequence (dashed lines in Fig. 3a). As mentioned above, the germ-line L10 flanking sequence lacks a complementary sequence to the  $J_1$  nonanucleotide signal sequence, GGTTTTTGT. However, regions I and II in Fig. 3a mark sequences where the L10 and  $J_1$  flanks are, at 12 out of 14, and 9 out of 12 sites respectively, homologous to inverted repeats of each other. It may be that the nonanucleotide signal sequences increase the probability of recombination, but are not indispensable. The sequences around the site of the L10- $J_1$  recombination may help to define the structural requirements of a putative recombinase. The same is true for another aberrant recombination which in a way is the inverse of the situation described here: in a variant of myeloma MPC 11 a  $V_\kappa$  gene segment has been found to have recombined to a DNA sequence between the  $J_\kappa$  cluster and  $C_\kappa$ <sup>17,18</sup> at the site of an heptanucleotide signal sequence<sup>18</sup> without an adjacent  $J$  gene segment.

The L10- $J_1$  joining is the first example of a  $J$  gene rearrangement in which the f-fragment persists in the same cell together with the reciprocal recombination product. In this case, therefore, one does not have to invoke sister chromatid exchange schemes, which explain well, by segregation, the non-reciprocity of f-fragments and  $\kappa$  genes found in other cases<sup>5,6</sup>. The L10- $J_1$  joining could be explained by a sister chromatid exchange model, but would require additional assumptions. An easy way to describe the L10- $J_1$  joining involves an inversion event (Fig. 3b). Note, however, that such a mechanism requires L10 and the  $J$  cluster to be orientated on the same chromosome with the opposite polarity, but the actual orientation is unknown. Inversion/deletion schemes, involving intrachromatid recombinations, have become attractive as they explain fairly easily an

aberrant recombination product of the heavy-chain  $J$  cluster<sup>19</sup>, and secondary  $\kappa$  gene rearrangements in a pre-B cell line<sup>7</sup>. Also, the rearrangement in the phase transition system of *Salmonella*, which uses signal sequences strikingly similar to those of the immunoglobulin genes, proceeds by an inversion<sup>20</sup>. As the location of f-fragments within the mouse genome is unknown, the possibility that DNA segments are excised and occasionally reinserted<sup>3</sup> cannot be ruled out.

The generation of all of the recombination products found so far can be explained by different combinations of inversion and deletion events, which has the merit of simplicity. However, the products of an inversion cannot be distinguished from those of a double, simultaneous sister chromatid exchange. Obviously, analyses of recombination products alone will not allow one to decide unequivocally between the mechanisms. Finally, although one may expect normal V-J rearrangements always to proceed by the same mechanism, it seems possible that different mechanisms could operate in the same cell, and that, in a given transformed cell, one or other mechanism may predominate. We are now investigating whether the L10 rearrangement is related to the rearrangements that have been found to join putative oncogenes to immunoglobulin gene regions in various B-lymphocyte malignancies (briefly reviewed in refs 21 and 22).

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## DNA rearrangements linked to expression of a predominant surface antigen gene of trypanosomes

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**African trypanosomes show an extensive capacity for antigenic variation<sup>1–6</sup>. The serotype of each antigenic variant is determined by a variant-specific surface antigen (VSA) which forms a continuous coat over the entire surface of the parasite<sup>7</sup>. All known VSAs are glycoproteins whose antigenic specificity is associated with the protein moiety<sup>8,9</sup>. The expression of some of the VSA genes is linked to the duplication of a 'basic' copy (BC) of the gene and to the transposition of the additional or 'expression-linked' copy (ELC) to a new site<sup>10–12</sup>, where it is transcribed<sup>13</sup>. Other VSA genes seem to be expressed without being duplicated; they are, however, subject to rearrangements apparently unrelated to their expression<sup>14,15</sup>. In chronic infections, different variable antigen types (VATs) tend to appear in a loosely ordered sequence: indeed, after either syringe passage or cyclical transmission through the tsetse fly, the same VATs always appear in the early stage of the disease and are therefore known as early or predominant ones<sup>16–22</sup>. To check whether genes coding for predominant VATs can be characterized by some particular feature of their DNA sequence or gene rearrangement, we have studied here the gene coding for AnTat 1.3, which is the most predominant VAT of our *Trypanosoma brucei brucei* stock. Three points emerged: (1) when expressed, the AnTat 1.3 gene is duplicated and the ELC transposed in an expression site identical to the one described previously for other VSA genes; (2) its BC is located near the end of a DNA molecule, in a region which is very similar to the expression site and undergoes frequent insertions/deletions unlinked to VSA gene expression; (3) this gene does not belong to a multigene family.**

To analyse the gene of the predominant antigen AnTat 1.3, we first prepared an AnTat 1.3-specific cDNA probe. Cloned populations of *T. b. brucei* (stock EATRO 1125) expressing the variable antigenic types AnTat 1.3 and AnTat 1.13 were obtained from infected rats as described previously<sup>19</sup>. Total poly(A)<sup>+</sup> RNAs were prepared from the parasites and used as template for the synthesis, by reverse transcription, of single- and double-stranded cDNAs<sup>23</sup>. The AnTat 1.3 double-stranded cDNA was then inserted in the *PvuII* site of the plasmid PBR322 by blunt-end ligation<sup>24</sup>, or in the *PstI* site of this plasmid by G–C tailing<sup>25</sup>. After transfection in *Escherichia coli* C600, AnTat 1.3-specific recombinants were selected by double screening<sup>23</sup> using the single-stranded AnTat 1.3 and AnTat 1.13 cDNAs as probes. This selection was then further checked

by hybridization of the putative AnTat 1.3 recombinant DNAs with Northern blots of poly(A)<sup>+</sup> RNAs isolated from several different antigenic variants<sup>23</sup>: this approach relies on the fact that a VSA-specific probe reveals transcription products in the homologous variants only, VSA genes being controlled at the transcription level<sup>9,10,23</sup>. Two recombinant plasmids were eventually retained, as their cDNA inserts, labelled with <sup>32</sup>P by nick-translation<sup>26</sup>, specifically revealed the 1.8-kilobase(kb)-long AnTat 1.3 mRNA (not shown). Restriction maps of these cloned cDNA inserts were constructed and have been included in Fig. 1. Their correct orientation with respect to transcription was established as described in Fig. 1 legend.

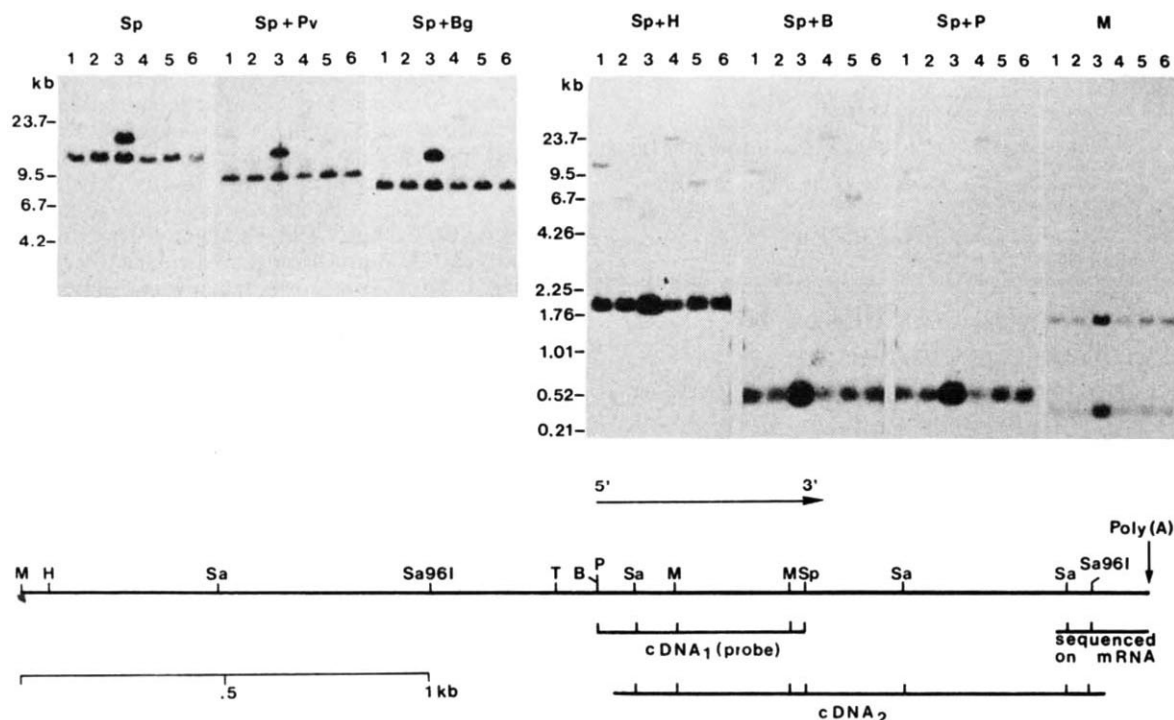
The 0.5-kb *PstI*–*SphI* AnTat 1.3 fragment of cDNA<sub>1</sub> (see Fig. 1) was then used as a probe to analyse genomic DNA blots prepared from a series of cloned populations of *T. b. brucei*, namely from variants AnTat 1.3, 1.1, 1.1b, 1.8 and 1.13, and also from procyclic trypanosomes derived *in vitro* from a clone expressing AnTat 1.1 (see ref. 12 for the pedigree of the different antigenic types).

If the genomic DNAs are digested by *SphI*, which cuts the cDNA sequence once, either alone or in conjunction with some other restriction enzymes (Fig. 1, digestions with *SphI*, *SphI* + *PvuII* or *SphI* + *BglII*), a single common AnTat 1.3-specific fragment is found in all the DNA blots and an additional one is clearly revealed in the homologous DNA, namely the DNA from trypanosomes synthesizing the AnTat 1.3 VSA (tracks 3).

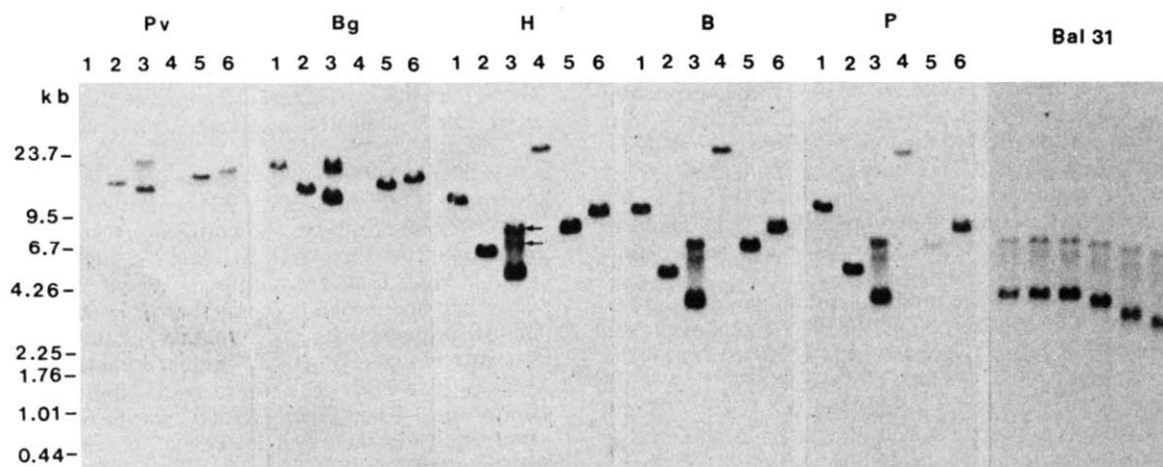
When the same genomic DNAs are cut either by one or a pair of enzymes which may generate internal fragments identified in the cDNA sequence (0.5-kb *SphI*–*PstI*, 0.5-kb *SphI*–*BglI* or 0.3-kb *MspI*), bands corresponding to these fragments are found in the genomic blots (Fig. 1, Sp + P, Sp + B and M), and their relative labelling intensity is increased in the AnTat 1.3 DNA (tracks 3). It is thus clear that an additional copy of the AnTat 1.3 gene is present in AnTat 1.3 DNA and that the expression of the 1.3 gene involves a duplication–transposition mechanism, as already demonstrated for other VSA genes<sup>10–12</sup>.

Digestion of the genomic DNA from non-expressors with either *PvuII*, *BglII*, *HindIII*, *BglI* or *PstI* (Fig. 2, tracks 1, 2, 4, 5, 6) yields a single band, which is obviously the sole candidate for the AnTat 1.3 BC. Surprisingly, however, the size of the fragment encompassing the BC differs from one variant to another, suggesting that it undergoes rearrangements of the type reported for the VSA genes whose expression does not depend on duplication–transposition<sup>14,15</sup>. Here also, an additional band is clearly seen in AnTat 1.3 DNA (Fig. 2, track 3 in Pv and Bg), reproducibly accompanied by a weaker third band and a smear after digestion by *HindIII*, *BglI* or *PstI* (Fig. 2, track 3 in H, B and P). Because it was not clear which band(s) contained the ELC, the latter was identified through its higher DNase I sensitivity (Fig. 3), and found to be present in the two upper bands indicated by small arrows in Fig. 2 (*HindIII* digestion).

We then constructed restriction maps extending over the environment of the AnTat 1.3 sequences present in each variant. These maps are shown in Fig. 4 and compared with similar maps of the AnTat 1.1 and 1.8 ELCs. The probable extent of the AnTat 1.3 transposed element (TE) is represented by the line under map c. Its size has been estimated from the minimal sequence length *l* that could account for the several internal restriction fragments present in both the BC and the ELC. For example, as shown in Fig. 1, the labelling intensity of both the 1.85-kb *SphI*–*HindIII* and the 1.6-kb *MspI*–*MspI* bands is clearly increased in the AnTat 1.3 DNA, indicating that they both contain inner fragments of the TE. Similarly, the 1.6-kb *Sau96I* and the 1-kb, 0.68-kb and 0.4-kb *Sau3A* fragments of the cDNA sequence are also inner fragments of the TE (not shown). This allowed us to attribute a size of at least 2.8 kb to the AnTat 1.3 TE; this size is larger than that of the mRNA (1.8 kb, see above): as observed for other TEs<sup>27–29</sup>, a stretch of DNA is thus co-transposed in front of the VSA gene.



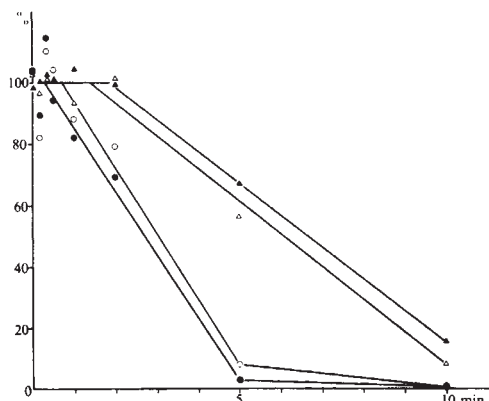
**Fig. 1** The AnTat 1.3 gene is duplicated and transposed when expressed. Nuclear DNA of cloned trypanosomes expressing VATs AnTat 1.1, 1.1b, 1.3, 1.13, 1.8 and a procyclic form derived from an AnTat 1.1 cloned population by *in vitro* subculturing (respectively in lanes 1–6), was digested in standard conditions<sup>23</sup> with the indicated restriction endonucleases and size-fractionated by electrophoresis through 0.5% (three blocks to the left) or 0.85% (four other blocks) agarose gels. Gels were blotted onto nitrocellulose filters, which were then hybridized with a <sup>32</sup>P-labelled probe made by nick-translation<sup>26</sup> of cDNA<sub>1</sub> (see map). <sup>32</sup>P-labelled *Hind*III digests of simian virus 40 (SV40) and  $\lambda$  DNAs provided the size markers. B, *Bgl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hi, *Hin*I; M, *Msp*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sa, *Sau*3A; Sa96I, *Sau*96I; Sp, *Sph*I; Ss, *Sst*I; T, *Taq*I; X, *Xor*II. The restriction maps, summarizing information collected from the two AnTat 1.3 cDNAs and from genomic DNA blots, illustrate how the probe is related to the AnTat 1.3 gene sequence and how the orientation of the maps with respect to transcription has been determined. The large map probably covers most of the transposed element, its 3' end corresponding to the polyadenylation site of the mRNA. Maps of two cDNAs are presented below: cDNA<sub>1</sub> was cloned by blunt-end ligation<sup>24</sup> and must be just a few base pairs larger than its 0.5-kb *Pst*I–*Sph*I inner fragment, while cDNA<sub>2</sub> was cloned by G-C tailing<sup>25</sup>. The 3' end of the AnTat 1.3 mRNA was sequenced by reverse transcription<sup>37</sup> of total AnTat 1.3 poly(A)<sup>+</sup> RNA primed by the oligonucleotide 5'-GGTGTAAATATATATCAG-3' (synthesized by Biolabs). This primer is complementary to a conserved sequence present next to the poly(A) track in the 3' end of all *T. b. brucei* VSA mRNAs examined so far<sup>38</sup>. The poly(A)<sup>+</sup> RNA (0.3  $\mu$ g) was annealed with 10 ng oligonucleotide primer in 10  $\mu$ l of 6 mM MgCl<sub>2</sub>, 60 mM NaCl, 20 mM dithiothreitol and 50 mM Tris (pH 8.3 at 42 °C) at 0 °C for 15 min. After the addition of 19 U of reverse transcriptase, 2- $\mu$ l aliquots were mixed with 2  $\mu$ l solution containing 100  $\mu$ M dTTP, dGTP and dATP, 3  $\mu$ M <sup>32</sup>P-dCTP (920 Ci mmol<sup>-1</sup>) and either of the four dideoxytriphosphates at 1/5th the concentration of the corresponding deoxytriphosphate. After incubating the mixtures at 42 °C for 30 min, 3  $\mu$ l of deionized formamide and 10 mM EDTA containing bromophenol blue and xylene cyanol FF were added. The samples were then heated at 95 °C for 3 min and applied to a 6% polyacrylamide gel in 8 M urea<sup>39</sup>. As indicated, the two restriction sites *Sau*96I and *Sau*3A mapped close to one end of cDNA<sub>2</sub> were found in this sequence, allowing correct orientation of the cDNA maps.



**Fig. 2** Further analyses of the AnTat 1.3 gene, indicating that it is located in a variable 3' DNA terminus. The 0.5-kb *Pst*I–*Sph*I probe was hybridized with Southern blots of genomic DNA isolated, as in Fig. 1, from antigenic variants AnTat 1.1, 1.1b, 1.3, 1.13 and 1.8 and of procyclic forms derived from a 1.1 (respectively in tracks 1–6 of the first five panels). Electrophoresis was in 0.5% agarose gels. The abbreviations for the restriction enzymes are as in Fig. 1. The last panel shows the kinetics of a *Bal*31 exonuclease digestion of AnTat 1.3 DNA. The DNA (10  $\mu$ g) has been incubated in 50  $\mu$ l of 0.1 mM NaCl, 12 mM CaCl<sub>2</sub>, 1 mM EDTA, 20 mM Tris-HCl (pH 8) with 1 U of *Bal*31 exonuclease, at 30 °C for 0, 2, 4, 6, 8 and 10 min, from left to right respectively. After extraction, the DNA was digested by *Pst*I and then analysed by Southern blotting in the same way as the other samples in the figure.



The most striking result of Fig. 4 is that the environment of the BC of the AnTat 1.3 gene (maps *d-i*) appears identical to the ELC's expression site (map *c*): like the ELC, the AnTat 1.3 BC is flanked on both sides by DNA stretches devoid of restriction sites. Still more significant, all the restriction sites that could be mapped upstream from the AnTat 1.3 BC (from left to right, *EcoRI*, *SphI*, *PvuII* and *BglII*) are also present in the 5' environment of the AnTat 1.3, 1.1 and 1.8 ELCs (maps *c*, *b* and *a*, respectively). Finally, all the restriction sites mapped downstream from the transposed element are clustered (Fig. 2, maps *d-i*), strongly suggesting that the 3' environment of the AnTat 1.3 BC is the end of a DNA molecule. This hypothesis



**Fig. 3** Sensitivity of the AnTat 1.3 sequences to DNase I digestion. To introduce adequate inner controls, the test was performed, according to Pays *et al.*<sup>13</sup>, on nuclei prepared from a mixture of cloned AnTat 1.3 and 1.1 trypanosomes. The DNA extracted from the nuclei after different incubation periods with DNase I was digested by *Hind*III, then transferred to nitrocellulose filters and hybridized with the same probe as for Fig. 1. The labelling intensity of the different bands was estimated by scanning with a Joyce-Loebl recording microdensitometer and plotted against incubation time, in per cent of the mean initial value. The 7.5-kb (○) and 6.3-kb (●) fragments of the AnTat 1.3 DNA (arrows in Fig. 2, H) are clearly more sensitive to DNase I than the AnTat 1.3 5.4-kb fragment (▲) or the AnTat 1.1 11.3-b fragment (△). It is inferred that the 7.5- and 6.3-kb fragments are both transcribed and contain the AnTat 1.3 ELC.

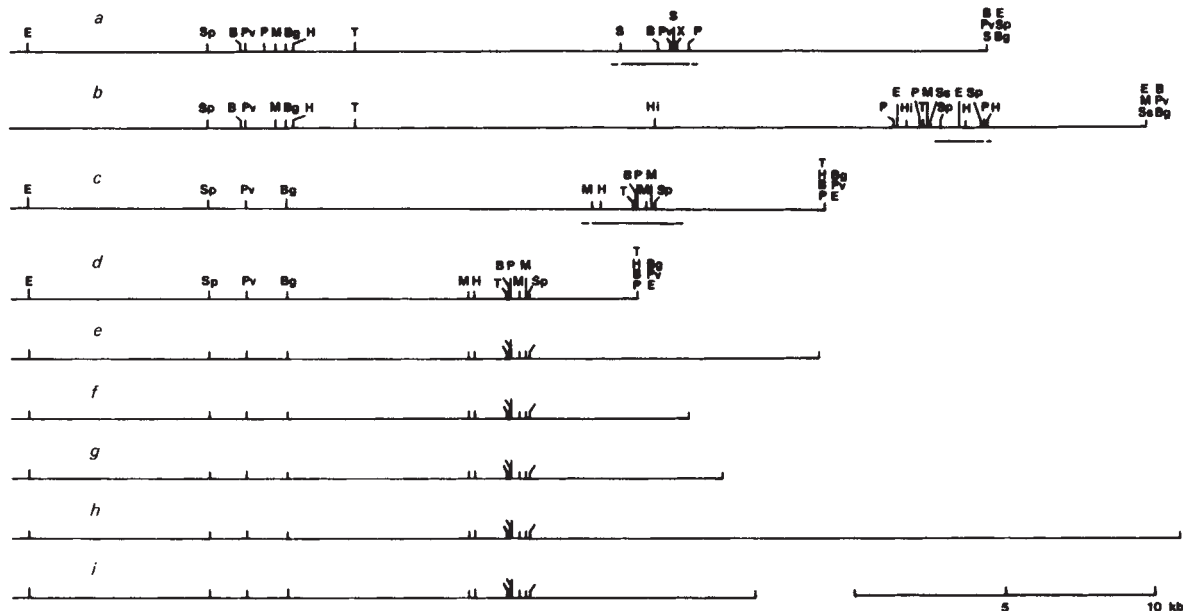
is strengthened by the fact that the AnTat 1.3 BC and ELC are both located in DNA regions progressively shortened by the exonuclease *Bal31* (Fig. 2).

It is no surprise that the AnTat 1.3 ELC is in a 3' DNA end: all ELCs studied so far are found within a long 'barren' stretch of DNA, probably made of simple repetitive sequences and located at the 3' end of a DNA molecule, probably of a chromosome<sup>29-31</sup>. We mentioned above that, in genomic blots obtained after *Hind*III, *Bgl*I or *Pst*I digestion, the ELC is present in at least two additional bands (presumably also in the accompanying smear, which is expression-linked) instead of the single additional one found after *Pvu*II and *Bgl*II digestion. Considering the relative positions of the probe (*Pst*I-*Sph*I) and of the different cleavage sites involved (see map c, Fig. 4), these observations are consistent with a model in which the AnTat 1.3 TE could be shuffled back and forth within the barren DNA region.

We found that the 5' environment of the AnTat 1.3 ELC appears identical to the corresponding region of the AnTat 1.1 and 1.8 ELCs: this extends previous observations and is in keeping with the view that the genome contains a single expression site for all the transposable VSA genes<sup>29,32</sup>. Because indirect evidence suggests a strong promoter located in the expression site, upstream from the transposed element, it has been proposed that the activation of at least some VSA genes may result from positioning its ELC downstream from this promoter<sup>29,33</sup>.

The existence of a non-expressed VSA gene within a 3'-terminal region, although unusual, has been described recently for the ILTat 1.3 and 1.4 VSA genes examined by Williams *et al.*<sup>30</sup> Like the two ILTat genes, the BC of the AnTat 1.3 gene is subject to insertions/deletions in its 3' surrounding. However, the AnTat 1.3 gene is duplicated when expressed, whereas the two ILTat ones are expressed according to an alternative and still unknown mechanism which does not involve the production of an ELC.

We have shown from restriction mapping that the 5' environment of the AnTat 1.3 BC is indistinguishable from the corresponding region of the expression site. Nevertheless, as transcription is restricted to the ELC, it is suggested that the 5' neighbourhood of the BC lacks the strong promoter supposed to be present in the expression site. One may further speculate



**Fig. 4** Restriction maps of the AnTat 1.3 and other VSA genes. *c* and *d* are, respectively, the restriction maps of the AnTat 1.3 ELC and BC in the genome of AnTat 1.3 trypanosomes. *e-i* are the AnTat 1.3 BCs found, respectively, in clones expressing the VATs AnTat 1.1, 1.1b, 1.8 and 1.13, and in procyclic trypanosomes derived from cloned AnTat 1.1 bloodstream forms by subculturing *in vitro*. Cleavage sites were mapped by hybridizing genomic DNA blots with the labelled *PstI-SphI* cDNA probe (see Fig. 1). Maps of the AnTat 1.3 sequences may be compared with those of the AnTat 1.8 and 1.1 ELCs (*a* and *b*, respectively). The minimal size of the transposed element is indicated by the thick line under maps *a*, *b* and *c*. The abbreviations for the restriction endonucleases are as in Fig. 1.

that, in the case of genes which, like the AnTat 1.3 gene, are in a chromosome extremity bearing considerable homology with the expression site, activation could be achieved by two alternative mechanisms: by the duplication-transposition process or by a reciprocal crossing-over which would allow the coding sequence to be positioned behind the transcription promoter without being duplicated. The view, expressed by Borst and Cross<sup>32</sup>, that genes activated without being duplicated are expressed very early in infection would thus be in accordance with this model, although the AnTat 1.3 case clearly shows that an early VAT may also be expressed by duplicative transposition. The predominance of a VAT like AnTat 1.3 is thought to result from a higher probability for it to be expressed<sup>19,34</sup>. In this context, the switch to AnTat 1.3 could be particularly favoured because the BC of the gene is in an environment which shares considerable sequence homology with the expression site, thus helping its translocation in the expression site.

At least four distinct but very similar 3'-terminal sequences have now been found in the same *T. b. brucei* stock: indeed, in addition to the AnTat 1.3 BC and ELC, two other sequences involved in antigenic variation have been identified in separate 3' DNA ends (E.P. *et al.*, in preparation). All four 3'-terminal sequences show length variability, most probably involving deletions and insertions within the barren sequences. With regard to the expression site, such alterations are generally linked to the insertion of a new ELC. In the case of the AnTat 1.3 BC, size alterations occur independently of AnTat 1.3 expression: as suggested by the results of the present work, they seem to occur, and could perhaps be induced, whenever an antigenic switch is taking place. This possibility is now being investigated. If confirmed, it would indicate that these rearrangements, including the switch itself, could be associated with and probably depend on the appearance of some control factor(s).

Only one genomic DNA band is clearly revealed by hybridization with the AnTat 1.3 probe at high stringency. It thus seems that, as opposed to the other VSA genes studied so far in the same stock<sup>12</sup>, the AnTat 1.3 gene does not belong to a multigene family. On the other hand, the AnTat 1.3 serotype has not been found in the other repertoires examined (including one of *Trypanosoma brucei gambiense*, four of *Trypanosoma brucei rhodesiense* and two of *Trypanosoma evansi*), except in one *T. b. brucei*. Both observations suggest that the AnTat 1.3 gene evolved recently, in obvious contrast with another VSA gene of the same repertoire, AnTat 1.8, which has a family, is widely distributed and is also remarkably conserved among trypanosome stocks and subspecies<sup>29,35,36</sup>. It is thus suggested that a new VSA gene could appear by frequent recombinations in an unstable 3'-terminal DNA environment, could then continue to evolve by further rearrangements and be shifted inside the chromosome until stabilization.

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## Expression of a bacterial modification methylase gene in yeast

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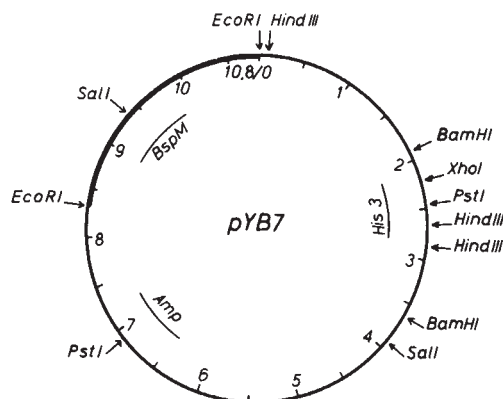
Methylation of specific cytosines in the DNA is generally believed to play some role in the regulation of gene expression in eukaryotes<sup>1-3</sup>. However, some eukaryotes, such as *Drosophila*<sup>4</sup> and yeast (S. Hattman, personal communication) seem not to contain 5-methylcytosine in their DNA. It would be interesting to test, how gene expression in such organisms would respond to the methylation of specific cytosines in the genome. As a first step towards this goal, we have introduced the gene encoding the *Bacillus sphaericus* R modification methylase, which methylates the internal cytosine within the recognition sequence 5'-GGCC, into yeast cells. Southern-type hybridization to DNAs isolated from the transformed yeast clones revealed that the yeast plasmid carrying the prokaryotic methylase gene, as well as the two chromosomal genes tested (*his3* and *leu2*) were methylated, whereas the bulk of the yeast DNA remained largely unmethylated. This indicates that the *Bacillus sphaericus* modification methylase was expressed in yeast but it modified only certain parts of the yeast DNA.

The gene coding for the modification methylase of *Bacillus sphaericus* R had previously been cloned in this laboratory and had been shown to be expressed in *Escherichia coli* from its own promoter<sup>5</sup>. The recombinant plasmid pES2 carries the methylase gene on a 2.5 kilobase (kb) *EcoRI* fragment inserted into the cloning vector pBR322. This fragment was ligated into the unique *EcoRI* site of the yeast-*E. coli* shuttle vector YEp6<sup>6,7</sup>. The recombinant plasmids isolated from *E. coli* HB101 were nondigestible by *BspRI*, indicating that they carried the functional methylase gene. Two such recombinants (pYB7 and pYB8; Fig. 1) which contained the 2.5 kb insert in opposite orientations, were selected for further study.

The histidine and leucine auxotrophic yeast (*Saccharomyces cerevisiae*) strain AH215 was transformed with pYB7 and pYB8, *his*<sup>+</sup> clones were selected and purified on minimal plates. DNA was prepared from such transformants and analysed for the presence of the *BspRI* methylase gene and its possible expression. Partial or full resistance of the isolated DNA to

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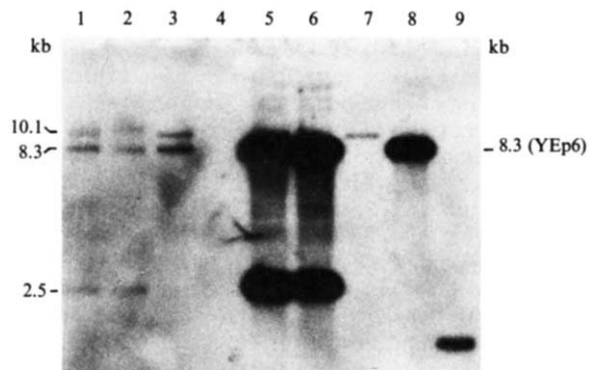


**Fig. 1** Map of the plasmid pYB7. YEp6<sup>6,7</sup> vector plasmid DNA (200 ng) was cleaved with *EcoRI*, treated with bacterial alkaline phosphatase and ligated to 400 ng of the 2.5 kb *EcoRI* fragment isolated from pES2<sup>5</sup>. *E. coli* HB101 was transformed with the ligated DNA and plasmid DNA was prepared<sup>8</sup> from 12 Ap<sup>R</sup> transformant clones. 9 plasmids carried the 2.5 kb methylase-fragment in the *EcoRI* site and were resistant to *BspRI* cleavage. The orientation of the insert was determined by *Sall* digestion.

*BspRI* cleavage was taken as evidence for the expression of the *BspRI* methylase. In the Southern-blotting experiment shown on Fig. 2 plasmid pYB7 extracted from yeast was not cleaved with *BspRI* although the 8.3 kb vector fragment is known to have several *BspRI* sites and was digested in the control experiment. The *his* probe used in this experiment identified in the yeast DNA a 10.1 kb hybridizing fragment which carries the chromosomal *his3* allele<sup>9</sup>. This fragment also appears to be protected in yeast transformed with the *B. sphaericus* methylase gene, but not in yeast transformed only with the vector. The vast majority of the cell DNA is, however, not protected as it could be seen on the ethidium bromide-stained gel (data not shown).

In another experiment (Fig. 3) the methylation of the *leu2* gene was tested. There was no selection for the function of this gene in the experiment. Southern hybridization shows (Fig. 3B) that in yeast cells transformed with pYB7, the YEp6 vector and the approximately 7 kb *BamHI* fragment of the chromosomal DNA carrying the *leu2* gene<sup>15</sup> are at least partially protected against *BspRI* cleavage, but they are cleaved in the control. The ethidium bromide-stained gel shows that majority of the yeast DNA was not protected.

These results demonstrate that the *BspRI* modification methylase is expressed in yeast and partially methylates the



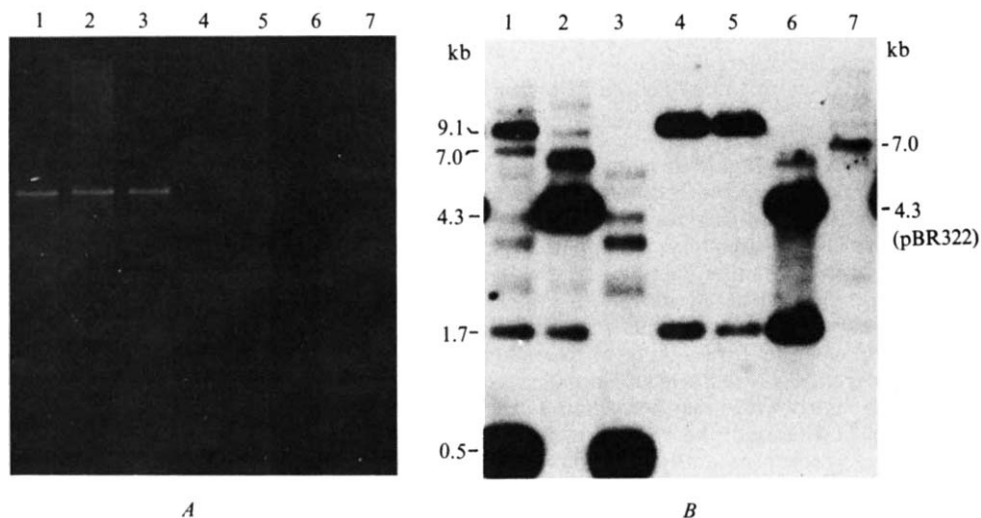
**Fig. 2** Testing by Southern-hybridization the methylation of the DNA isolated from transformed yeast. Slot 1, yeast, transformed by pYB7, *EcoRI* digestion; 2, yeast, transformed by pYB7, *EcoRI* + *BspRI* digestion; 3, yeast, transformed by YEp6, *EcoRI* digestion; 4, yeast, transformed by YEp6, *EcoRI* + *BspRI* digestion; 5, pYB7 plasmid, *EcoRI* digestion; 6, pYB7 plasmid, *EcoRI* + *BspRI* digestion; 7, Untransformed yeast (AH215), *EcoRI* digestion; 8, YEp6 plasmid, *EcoRI* digestion; 9, YEp6 plasmid, *EcoRI* + *BspRI* digestion.

**Methods:** Yeast strain AH215 (*a*, *his3*-11, *his3*-15, *leu2*-3, *leu2*-112, *can1*-101) was transformed<sup>10,11</sup> with pYB7 or YEp6 plasmid. His<sup>+</sup> transformants were isolated and grown on YNB plates (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 30  $\mu$ g ml<sup>-1</sup> leucine, 2% agar). DNA prepared<sup>6</sup> from yeast cells collected from the plates was digested with *EcoRI* or with *EcoRI* and *BspRI* and analysed by electrophoresis on 0.8% agarose gel. Yeast (3  $\mu$ g), pYB7 (30 ng) or YEp6 plasmid DNA (10 ng) was applied. Plasmids in lanes 5, 6, 8 and 9 were isolated from *E. coli* HB101. After electrophoresis the DNA was blotted to nitrocellulose<sup>12</sup> and hybridized in a solution containing 2 $\times$ SSC, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 50  $\mu$ g ml<sup>-1</sup> sheared denatured chicken blood DNA, 1% SDS at 65°C for 16 h with pES2 and pBR322/*his3* (the latter plasmid carries the yeast *HIS3* gene on a 1.7 kb *BamHI* fragment in pBR322 vector<sup>13</sup>) probes labelled with <sup>32</sup>P by nick-translation<sup>14</sup>. The filters were washed in 1 $\times$ SSC at 65°C for 1 h and autoradiographed.

host DNA, rendering it resistant to *BspRI* cleavage. The *BspRI* resistance cannot be explained by assuming the existence of a host maintenance methylase activity because in addition to the introduced pYB7 plasmid, also the chromosomal *his3* and *leu2* genes (and their immediate surroundings) appear to be protected. Thus the *BspRI* methylase gene acts in *trans*. This was further confirmed by cotransformation of yeast with pYB7 and another plasmid pJDB207<sup>16</sup>: in double-transformants the

**Fig. 3** Testing of the methylation of the *leu2* gene in transformed yeast. A, Ethidium bromide-stained gel; B, autoradiogram after Southern-blotting and hybridization. Slot 1, yeast, transformed by pYB7, *BamHI* + *BspRI* digestion; 2, yeast, transformed by YEp6, *BamHI* digestion; 3, yeast, transformed by YEp6, *BamHI* + *BspRI* digestion; 4, pYB7 plasmid, *BamHI* digestion; 5, pYB7 plasmid, *BamHI* + *BspRI* digestion; 6, pBR322/*his3* plasmid, *BamHI* digestion; 7, untransformed yeast (AH215), *BamHI* digestion.

**Methods:** The following amounts of DNA were applied: 10  $\mu$ g of yeast DNA, 10 ng of pYB7 and 10 ng of pBR322/*his3*. Plasmids in lanes 4, 5, 6 were isolated from *E. coli* HB101. Samples in slots 1-3 contained 10 ng of pBR322 DNA in order to control the completeness of the *BspRI* digestion. The bright fluorescent bands (lanes A1-3) are endogenous double-stranded RNA. The conditions of the electrophoresis, transfer and hybridization were the same as in the experiment shown on Fig. 2 except that the probe contained in addition to pES2 and pBR322/*his3* also pBR322/*leu2* plasmid which carries the yeast *LEU2* gene on an approximately 7 kb *BamHI* fragment in pBR322. In this experiment protection of the chromosomal *his3* gene cannot be seen because the size of the chromosomal *his3* fragment is the same as that of the pYB7 *his3* fragment.



pJDB207 became partially resistant to *Bsp*RI (data not shown). The expression of the bacterial methylase gene seems to be dependent on the bacterial promoter because expression was observed in both orientations of the insertion (results obtained with pYB8 were similar to those of pYB7).

There is striking difference between the low overall protection of the cell DNA and the high-level protection of the plasmids and the two chromosomal genes investigated in this study. We explain this with nonrandom methylation. It is tempting to speculate that the extent of methylation may depend on chromatin structure. Further investigation is needed to learn more about the nature of the DNA regions, which become methylated, and about the factors governing this process. It is possible that this methylation may be a useful *in vivo* probe of chromatin organisation.

To our knowledge this is the first example of a gene from a Gram-positive bacterium shown to be expressed in yeast. As the sequence of the *Bsp*RI methylase gene and its promoter is known (G. Pósfai *et al.*, manuscript in preparation) this might help explain the requirements of such heterologous gene expression. This is also the first example of the introduction of a 'foreign' site-specific methylation activity into an eukaryotic cell. As yeast does not contain endogenous cytosine-methylase (that is its DNA is unmethylated) the effect of the new activity, the pattern of methylation can be conveniently studied in this host.

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## Novel lipid components of the *Azotobacter vinelandii* cyst membrane

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Phospholipids are ubiquitous components of biological membranes. In the vegetative cells of *Azotobacter vinelandii*, a Gram-negative free-living aerobic soil bacterium, the membrane lipids are phospholipids with polar head group and fatty acyl compositions similar to those of *Escherichia coli*<sup>1</sup>. We report here that when *A. vinelandii* differentiates to form metabolically dormant cysts, the phospholipids in the membranes are replaced by a family of 5-*n*-alkylresorcinols and 6-*n*-alkylpyrones. These novel amphiphilic lipids form a unique membrane matrix which may contribute to the physiology and desiccation resistance of the cyst.

Encystment occurs naturally in less than 1% of late stationary *A. vinelandii* cells; however the yield of cysts can be increased to over 90% by culturing log-phase cells in *n*-butanol or  $\beta$ -hydroxybutyrate<sup>2</sup>. The encystment process takes 5 days to complete and results in a spherical phase-bright resting cell (central body) surrounded by a modified capsule (intine) and a layered outer shell (exine) (Fig. 1). The transition from vegetative cell to cyst has been studied enzymologically<sup>3</sup>, by electron micro-

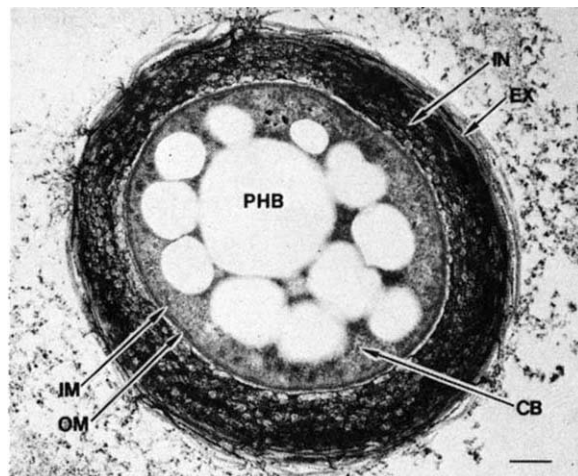


Fig. 1 Electron micrograph of a thin section of a mature cyst of *A. vinelandii*. Note the exine (EX), intine region (IN), the inner (IM) and outer membrane (OM) of the central body (CB), and the large accumulation of polyhydroxybutyrate (PHB). Scale bar, 0.2  $\mu$ m.

scopy<sup>4</sup> and by changes in lipid composition<sup>5</sup>. Lipids are the major metabolic products of the cell during encystment. We have previously reported that after the induction of encystment, phospholipid synthesis is curtailed, extant phospholipid is degraded, and D(-)poly- $\beta$ -hydroxybutyrate is rapidly accumulated in large phase-bright granules. Then (after 6-8 h) the cells begin the synthesis of 5-*n*-heneicosylresorcinol (AR<sub>1</sub>) followed by the remainder of the family of 5-*n*-alkylresorcinols and 6-*n*-alkylpyrones (see Fig. 2 for structures). The central body of the mature cyst contains 16% (dry weight) of resorcinols and pyrones and less than 1% (dry weight) of phospholipid<sup>5</sup>.

Cysts can be germinated by culturing them in glucose or other carbohydrates. The transition occurs over 12-15 h, and is followed by a resumption of vegetative growth. During this time, AR and AP synthesis does not occur, and phospholipid synthesis resumes (unpublished data).

Examination of electron micrographs of cysts reveals that the central body has an inner membrane (IM) and an outer membrane (OM) (Fig. 1). The absence of significant quantities of phospholipid in the cyst, together with the amphiphilic nature of resorcinols and pyrones suggested to us that the 5-*n*-alkylresorcinols and 6-*n*-alkylpyrones could be constituents of these membranes. Therefore, we isolated IM and OM fractions of cysts by sucrose density gradient centrifugation (Fig. 3) and determined their lipid composition. We found that the ARs and APs constitute 95% of the chloroform-methanol-extractable lipids in the central body membranes. The lipid compositions of the IM and OM are the same, within experimental error, in both cysts and vegetative cells. Average values are given in Table 1. The small amount of phospholipid (5%) found in the cyst membrane may represent an essential minimum requirement for membrane stability, or may be a consequence of incomplete encystment.

We calculate from measurements taken from electron micrographs that the vegetative cell has an average volume of 13  $\mu$ m<sup>3</sup> and an average surface area of 29  $\mu$ m<sup>2</sup>. For the central body of the cyst these values are 2  $\mu$ m<sup>3</sup> and 8  $\mu$ m<sup>2</sup> respectively. Because the densities of the cyst central body and the vegetative cell are approximately the same<sup>6</sup>, we may assume that the dry weights are proportional to the volumes, and therefore that the central bodies have approximately 1.7 times as much surface area per unit dry weight as the vegetative cell. As the vegetative cell has 9.2% lipid and the central body has 16% lipid (exclusive of poly- $\beta$ -hydroxybutyrate)<sup>5</sup>, the weight of lipid per unit surface area is approximately the same in the vegetative cell and the cyst central body.

The average molecular weight of the phospholipids in the vegetative cell is 758, and that of the cyst lipids is 457. As each



**Table 1** Lipid content of membranes

| Lipid                             | Vegetative cell membrane*<br>(% of total lipid) | Cyst membrane*<br>(% of total lipid) |
|-----------------------------------|-------------------------------------------------|--------------------------------------|
| <b>Phospholipids</b>              |                                                 |                                      |
| PE                                | 80                                              | 1                                    |
| PG                                | 14                                              | 1                                    |
| CL                                | 3                                               | 1                                    |
| PS                                | 1.5                                             |                                      |
| Others                            | 1.5                                             | 2                                    |
| <b>Resorcinols</b>                |                                                 |                                      |
| AR <sub>1</sub>                   | —                                               | 25                                   |
| AR <sub>2</sub>                   | —                                               | 25                                   |
| AR <sub>3</sub> + AR <sub>5</sub> | —                                               | 8                                    |
| AR <sub>4</sub>                   | —                                               | 18                                   |
| <b>Pyrones</b>                    |                                                 |                                      |
| AP <sub>1</sub>                   | —                                               | 11                                   |
| AP <sub>2</sub>                   | —                                               | 4                                    |
| Others                            | —                                               | 4                                    |

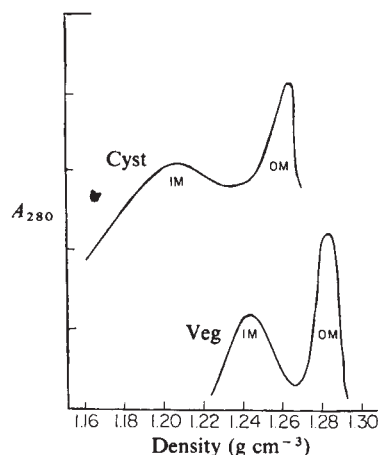
Cells were grown vegetatively at 30 °C with 48 mM glucose containing [2-<sup>14</sup>C]glucose (0.41  $\mu$ Ci mM<sup>-1</sup>) and were encysted with 16 mM sodium  $\beta$ -hydroxybutyrate containing [3-<sup>14</sup>C] $\beta$ -hydroxybutyrate (0.41  $\mu$ Ci mM<sup>-1</sup>). Membranes were isolated as described in the legend to Fig. 3. Lipids were extracted from washed membrane fractions as previously described<sup>8</sup>. Phospholipids, alkylresorcinols and alkylpyrones were separated by two-dimensional thin-layer chromatography on TLC silica gel 60 aluminium sheets. Development in the first direction was with chloroform/methanol/water (65:25:4) and in the second direction with chloroform/methanol (85:15). Lipids were detected with I<sub>2</sub> vapour. The relative amounts of the lipids was determined from their relative radioactivity, measured by scintillation counting (ACS:H<sub>2</sub>O; 90:10) in a Packard Tri-Carb liquid scintillation spectrometer. PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine.

\* Values are an average two determinations each for IM and OM.

| Structure | Lab name        | R                                                                  | Ratio    |
|-----------|-----------------|--------------------------------------------------------------------|----------|
|           | AR <sub>1</sub> | C <sub>21</sub> H <sub>43</sub><br>C <sub>23</sub> H <sub>47</sub> | 87<br>13 |
|           | AR <sub>2</sub> | C <sub>21</sub> H <sub>43</sub><br>C <sub>23</sub> H <sub>47</sub> | 87<br>13 |
|           | AR <sub>3</sub> | C <sub>21</sub> H <sub>43</sub><br>C <sub>23</sub> H <sub>47</sub> | 1<br>1   |
|           | AR <sub>4</sub> | C <sub>19</sub> H <sub>39</sub><br>C <sub>21</sub> H <sub>43</sub> | 3<br>4   |
|           | AR <sub>5</sub> | C <sub>21</sub> H <sub>43</sub><br>C <sub>23</sub> H <sub>47</sub> | 6<br>4   |
|           | AP <sub>1</sub> | C <sub>21</sub> H <sub>43</sub><br>C <sub>23</sub> H <sub>47</sub> | 2<br>5   |
|           | AP <sub>2</sub> | C <sub>19</sub> H <sub>39</sub><br>C <sub>21</sub> H <sub>43</sub> | 1<br>1   |

**Fig. 2** Structures of the lipids isolated from *A. vinelandii* cyst membranes.

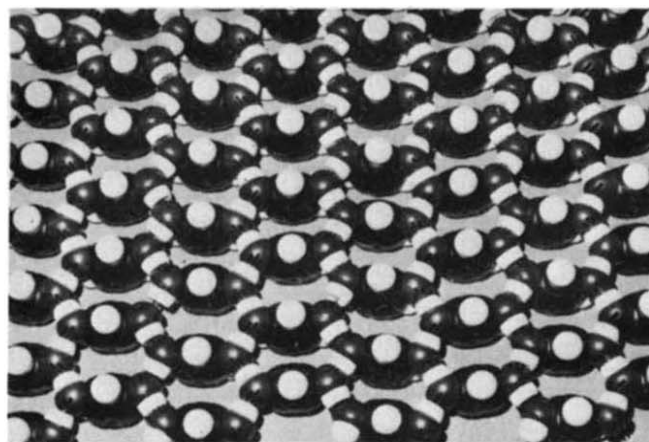
phospholipid molecule has two fatty acyl chains, and each resorcinol and pyrone molecule has one polymethylene chain, the density of the hydrocarbon chains in the central body membranes is 85% that of the vegetative membrane. This



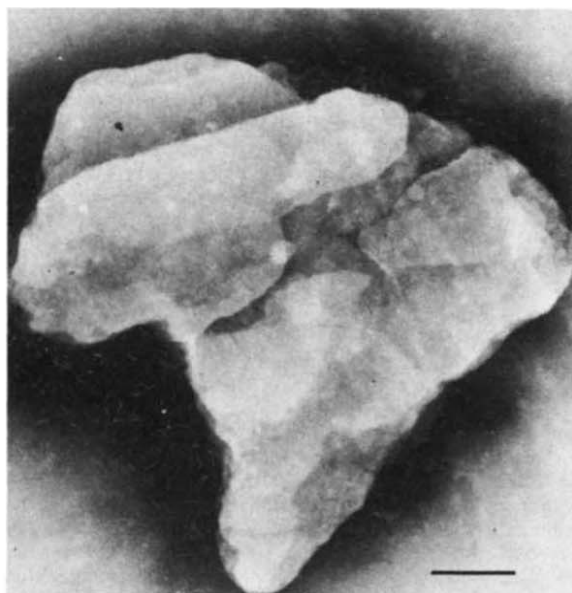
**Fig. 3** Protein profiles of vegetative (veg) and cyst membranes fractionated by sucrose density gradient centrifugation. The absorbance at 280 nm is contributed to by the resorcinols and pyrones, which have  $\lambda$  max of 275 nm and 278 nm and log  $\epsilon$  of 3.25 and 4.09 respectively. **Methods:** Membrane fractions of *A. vinelandii* ATCC 12837 were isolated essentially as described by Smit *et al.*<sup>9</sup>. Vegetative cells and cysts were suspended in cold 10 mM HEPES, pH 7.4, containing ribonuclease A and deoxyribonuclease 1 (1 mg per 20 ml suspension) and the suspension was passed three times through a cold French pressure cell (~8,000 p.s.i.). Unbroken cells were removed by centrifugation at 2,000g for 10 min. Total membranes were sedimented onto a layer of 65% sucrose by centrifugation at 28,000 r.p.m. (Spinco 30 rotor) for 2 h, collected and layered onto linear sucrose gradients (28–62% in 10 mM HEPES, pH 7.4). Gradients were centrifuged at 25,000 r.p.m. (Spinco SW27 rotor) for 40 h. Membrane fractions were collected by density displacement with a density gradient fractionator (ISCO model 640) with continuous monitoring of protein by absorbance at 280 nm. There was considerable cross-contamination of IM and OM (up to 25% in cysts) based on ketodeoxyoctonate (KDO) content per dry weight of membrane. Cyst IM was identified by relative absence of KDO, by density and by electron microscopic examination. IM isolated from vegetative cells and cells at progressive stages of encystment showed a gradual decrease in density and loss of enzymatic activity (unpublished data). NADH oxidase, succinate dehydrogenase and lactate dehydrogenase<sup>10</sup> activities diminished rapidly after induction of encystment and are not detectable after 12 h. NADH oxidase was used as an enzyme marker for vegetative IM, and KDO<sup>11</sup> as a chemical marker for vegetative and cyst OM. Cyst OM was also examined by EM.

difference in densities of cyst and vegetative membrane lipids is reflected in the densities of their IM and OM (Fig. 3).

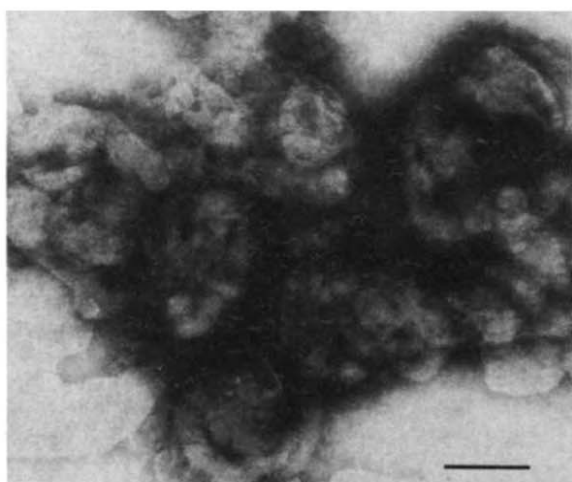
The structures of the cyst lipids differ appreciably from those of the vegetative phospholipids. The phospholipids have two fatty acyl chains per head group, 62% of which are unsaturated, whereas the cyst lipids have single saturated hydrocarbon chains which are connected directly to an aromatic (alkylresorcinol) or pyrone (alkylpyrone) ring. The hydrocarbon chains of amphiphilic molecules can assume a stretched all-*trans* conformation in which they form hydrophobic cylinders with a cross-sectional area of approximately 21 Å<sup>2</sup>, and an average chain length of 1.3 Å per methylene residue with the addition of the van der Waals' radius of the terminal methyl group (1.5 Å). The methylene group proximal to a polar group is excluded as it does not contribute appreciably to the hydrophobic region<sup>7</sup>. For the phospholipids of *A. vinelandii* the fatty acyl chains have an effective average length of 19 Å and average volume of 400 Å<sup>3</sup>. The augmented polymethylene chains of the cyst lipids average 27 Å in length and 570 Å<sup>3</sup> in volume. This increased hydrophobic area promotes their aggregation. The polar heads of the cyst lipids are smaller than those of the phospholipids. Measurement made with Corey–Pauling space-filling molecular models indicate a head length of 5.5 Å, compared with 8 Å for the phospholipids. The membrane surface structure is extended by the hydration of the polar heads,



a



b



c

**Fig. 4** *a*, Top view of a model of the polar head groups of AR<sub>1</sub> showing the staggered-chain structure formed by hydrogen-bonding between *meta*-hydroxyl groups. *b*, Electron micrograph of structures formed by ultrasonic dispersion of AR<sub>1</sub> in water. Negative staining with 2% potassium phosphotungstate. Scale bar, 0.1  $\mu$ m. *c*, Electron micrograph of structures formed by ultrasonic dispersion of total cyst membrane lipids in water stained as in *b*. Scale bar, 0.1  $\mu$ m.

and for charged head groups by the presence of hydrated counterions. This hydrated region would be larger for the anionic and zwitterionic phospholipids than for the uncharged alkylresorcinols and alkylpyrones. When all these factors are

considered, the width of the membrane bilayer should be approximately the same in the cyst and vegetative cell, although the ratio of hydrophobic to hydrophilic regions is greater in the cyst membrane.

The formation of micelles or bilayers by amphiphilic molecules requires a balance between the forces of self-association and repulsion<sup>7</sup>. As the hydrophobic effect of the long saturated polymethylene chains results in self-association, the repulsive force must come primarily from the interaction of the polar head groups. In uncharged amphiphilic molecules, such as the alkylresorcinols and alkylpyrones, this repulsion is primarily steric, and requires a preference for hydration rather than association. As the hydroxyl groups of the alkylresorcinols and alkylpyrones can act both as hydrogen bond donors and hydrogen bond acceptors, the head groups of the cyst lipids may associate. The first cyst lipid synthesized, AR<sub>1</sub>, has *meta*-hydroxyl groups which allow it to self-associate forming a staggered-chain conformation in which the polar head groups have hexagonal symmetry (Fig. 4*a*). In this closest packing configuration the effective surface area per head group is 34 Å<sup>2</sup> (measurements made in model structures). The chains would be packed hexagonally in the all-*trans* configuration with the chain axis at an angle of 109° to the head group. When a monolayer of AR<sub>1</sub> on water is compressed to this surface area per molecule in a Langmuir trough the surface loses its transparency and assumes a crystalline-like appearance. The other major cyst lipids contain only one hydroxyl group for association. They have other hydrophilic groups, such as the carbohydrate moiety of AR<sub>2</sub>, and the carbonyl and ring oxygens of the pyrones which require hydration. The introduction of these lipids interrupts the paracrystalline structure of AR<sub>1</sub> and provides the repulsive force that prevents the separation of these lipids into a distinct phase, and allows the formation of a stable bilayer for the cyst membrane.

Electron microscopic examination of the structures formed by ultrasonic dispersion of these lipids in aqueous media supports these conjectures. AR<sub>1</sub> forms layered sheets (Fig. 4*b*) consistent with the model structure (Fig. 4*a*), whereas total alkylresorcinols and alkylpyrones form rounded vesicular structures (Fig. 4*c*).

The amphiphilic lipids of the *A. vinelandii* cyst membranes differ from the phospholipids in many important ways. They form a membrane with a relatively narrow and uncharged polar region and a wider but less dense hydrophobic region. Their saturated polymethylene chains have higher melting temperatures, and are consequently more rigid at physiological temperature than the fatty acyl chains of phospholipids. The alkylresorcinols and alkylpyrones constitute a very hydrophobic and viscous membrane structure which may contribute to the desiccation resistance, dormancy and other properties of cysts. The membranes of the *A. vinelandii* cyst may be useful as a matrix for studying the insertion of phospholipid molecules into membranes, phospholipid flipping from one leaflet of a bilayer to another, phospholipid activation of membrane proteins and membrane transport. Our understanding of the role of phospholipids in membranes may be extended by studies of this novel phospholipid-free membrane.

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## Electromagnetic jet propulsion: non-lorentzian forces on currents?

TWO instances have been reported by Graneau<sup>1</sup> of an electromagnetic force on a current element apparently acting in the direction of current flow, in contrast to the generally accepted Lorentz force which always acts at right angles to the current, and he believes that these observations support instead Ampère's older formula for the force acting between two current elements.

In the first place, however, both observations can be understood in terms of Lorentz forces, for in the examples given, the current flow lines diverge from a point of constriction, so that the locally generated magnetic field exerting a force normal to the current produces a component of force parallel to the axis of symmetry of the diverging current, directed away from the apex. A quantitative check is possible in the case of the 'copper submarine', the long copper rod (radius  $a = 1.5$  mm), blunt at one end but tapered to a point at the other, which submerged itself when placed in a trough of mercury (0.25 sq. inch cross-section) carrying a 400 A current ( $I$ ), and travelled along the trough at a speed of  $15 \text{ cm s}^{-1}$ , always with the pointed end trailing.

Because of the high conductivity of copper, much of the current will be funnelled into the copper not far from the point, from where the lines of current flow will spread apart somewhat on moving towards the wider part of the copper, and in this cone section the Lorentz force has a component parallel to the axis, the magnetic field lines being circles around the axis. (After leaving the blunt end, the current will again diverge, exerting a propulsive force on the mercury.) Taking the resistance per unit length of the mercury to be  $k$  ( $\approx 2.67$ ) times that of the copper at its full width, and assuming that in any plane normal to the central axis the current density is nearly uniform in each medium and in proportion to conductivity, the Lorentz forces can be integrated over the whole of the cone section of the copper, to give the total longitudinal force  $\mu_0 I^2 F / 4\pi$  (SI units), where  $F = \frac{1}{2} \{ \ln(1+k) - (k/1+k) - \frac{1}{2}(k/1+k)^2 \}$ , directed away from the apex. The motion is resisted essentially by the inertial force  $\approx \rho v^2 \pi a^2$  (neglecting any motion of the fluid),  $\rho$  being the density of mercury. This gives a velocity  $v = 0.16 \text{ m s}^{-1}$ , in good agreement with observation.

In the other observation cited, where mercury flowed away from the end of a stationary wire carrying a current, the current would fan out into the mercury and so exert a force near the point of divergence, directed away from the wire.

Second, one cannot distinguish by experiment between the two different laws for the mutual forces acting on two

current elements, for one can observe only the total force acting on a current element, and not which element is the source of this force. It was established long ago<sup>2,3</sup> that both laws yield identical results for the force on a current element exerted by a current which flows in a complete circuit.

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GRANEAU REPLIES—Hillas believes that the motive force in the two experiments is being provided by certain components of the Lorentz force on the hair-pin bend and the conical part of the submarine. These components have no corresponding equal and opposite reaction forces in the liquid mercury or any other material medium. They may be termed self-forces, giving rise to self-propulsion. In the strict sense of special relativity theory, they are endowed with reaction forces in the field (vacuum) which accelerate matterless electromagnetic mass. Forces on vacuum may not be counted towards the force balance required by Newton's third law. On this issue Vannevar Bush<sup>2</sup>, Cleveland<sup>3</sup>, O'Rahilly<sup>4</sup>, Whittaker<sup>5</sup> and Robertson<sup>6</sup> aligned themselves with the newtonian dynamics. Their ranks must have swollen considerably since space travel has become a reality, for nobody tries to exploit vacuum reaction forces in pursuit of novel methods of space propulsion.

Hillas' references to Maxwell and O'Rahilly concern the analysis of rectangular circuits and not arbitrarily shaped, asymmetrical circuits. It is an accident of symmetry that the self-forces on the two parts of a rectangular circuit are equal and opposite and of the same magnitude as the Ampère reaction forces. For an analysis of unsymmetrical circuits of any shape one has to go back to the writings of Ampère<sup>1</sup>.

Hillas is correct in maintaining that the Ampère and the Lorentz force on an isolated current-element are the same when produced by the current in a separate closed circuit. But this is not true when the isolated current-element is incorporated in the closed circuit. Instead of relying on Ampère's long analysis, this can today be verified by finite current-element analysis with computers. It will then be found that, around an arbitrarily shaped circuit, the Ampère force has everywhere a tangential component in addition to the transverse component, the latter being equivalent to the Lorentz force. The tangential or longitudinal Ampère forces are consistent with the generation of

tension in straight wires and jet-reactions at liquid–solid conductor interfaces.

The Lorentz force was tailored to the electron moving through vacuum. No compelling argument seems to have been made for the moving free electron to be the same entity as an amperian metallic current-element. Perhaps there is room for two force laws?

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## The significance of transient haematopoietic spleen colonies

RECENTLY new and interesting observations have been reported by Magli *et al.*<sup>1</sup>, which led them to question the validity of the widely used 'spleen colony' assay for multipotential stem cells in blood forming tissues. Their observation was that the colonies of cells in the spleen 7 days after transplantation of bone marrow into irradiated mice were only transient, and so cannot be considered as measuring cells with extensive proliferative capacity. Their conclusion has been reached through insufficient attention being given to existing experimental data and failure to distinguish carefully between the capacity of the cells (their nature) and the particular demands made upon them (their nurture).

Many years ago Siminovitch *et al.*<sup>2</sup> pointed out that if differentiation was a stochastic event there should be a wide variation in the number of stem cells in colonies derived from single cells and he reported measurements that fitted the expected highly skewed distribution. Later Vogel *et al.*<sup>3</sup> estimated the 'extinction' coefficient, that is, the proportion of stem cells reaching the spleen which differentiate before being able to establish a colony. Therefore some stem cells will fail to produce any colonies, while others can be expected to run out of stem cells later, thus producing transient colonies, caused by the pressure of differentiation in the spleen, without the cells necessarily lacking self-renewal capacity.

A further aspect concerns the competing demands for stem cells to maintain both their own cell numbers and provide cells for differentiation. It is known that the population of cells measured by the 'spleen colony' assay and scored at 7 days is heterogeneous, comprising cells ranging from those with low proliferation rate and high capacity for self-renewal to cells with

a high proliferation rate and low self-renewal<sup>4,5</sup>. Recently, experimental data have been reported suggesting that only the proliferating stem cells are able to respond to the stimulus for differentiation and that this stimulus is part of the feedback controlling the number of stem cells<sup>6</sup>. The heterogeneity of stem cells can be explained on the basis of this hypothesis by postulating that stem cells which have divided recently are more likely to do so again and are more sensitive to the stimulus for differentiation. Accordingly, the rapidly proliferating stem cells with high sensitivity to the stimulus for differentiation and therefore low self-renewal capacity, could supply most of the cells for differentiation; while the slowly proliferating stem cells will be unlikely to differentiate and therefore provide a reserve population. In the event of a shortage of stem cells, however, the stimulus for differentiation must decrease so that many stem cells no longer differentiate and thus can no longer be considered to be lacking in self-renewal capacity. The extent to which stem cells are called upon to self-renew will therefore depend on the level of homeostatic control operating and this will alter the distribution of stem cells with differing proliferation and self-renewal capacity. Thus the composition of cells within developing or vanishing spleen colonies would appear to be as much due to the exigencies of the spleen colony assay as to any stem cell inadequacy.

To conclude, therefore, it is an oversimplification to suggest that stem cells scored at less than 11 days do not measure cells with extensive proliferative capacity. Furthermore these cells may well have a major role in the maintenance of haematopoiesis, even though some of them are not capable of long-term haematopoiesis. A more comprehensive view is needed of the 'nature' of stem cells and their 'nurture' as a result of the homeostatic control, together with experimental data demonstrating whether the differentiation and self-renewal observed in the spleens of the assay mice reflects what they do in the tissue from which they were taken. Until such information is available it is premature to question the validity of much of the work done in this field over the past 20 years, and restrict the counting of colonies until later than 11 days, with the attendant problems of reduced mouse survival and reduced number of colonies that can be counted on each spleen.

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**ISCOVE ET AL. REPLY**—Our experiments confirmed previous work in showing that day 7-8 spleen colonies are mainly erythroid and lack primitive precursors, whereas most 11-12 day-old colonies are multilineal and do contain primitive precursors. Because similar numbers of macroscopic colonies are found at early and late time points, it was assumed in the earlier literature that early and late colonies were the same colonies, and that their differing compositions reflected changes in local regulatory influences with time. If the assumption of identity between early and late colonies was correct, then properties of pluripotentiality and renewal capacity had to be ascribed to the cells originating colonies scored at earlier time points as well as to those forming the later colonies. Hence the widespread acceptance of the view that spleen colonies are a measure of pluripotential precursors with some renewal capacity regardless of when they are scored.

The significant point made by our study was that early macroscopic colonies are transient and are not the forerunners of the later colonies. We are left then with the observations that the early colonies are not multilineal and do not contain

### Tiltmeter recordings re-analysed for earthquake precursors

HERE I aim to qualify the conclusions of a paper by Gerard<sup>1</sup> which appeared to give good evidence for precursory earth tilt preceding earthquakes near Wellington, New Zealand.

Gerard was very conscious of the need to allow the equipment to settle long enough before attempting to interpret the tiltmeter recordings. He also used statistical procedures to assess and allow for the tilts induced by rainfall and other meteorological factors. The long-term trend was removed and the resulting tilt departures in the north-south and east-west directions were displayed on a polar plot. Two groups of points lay well outside the 1- $\mu$ rad diameter circle, preceding and around the times of earthquakes having magnitudes of 5.5 and 6.2.

In this laboratory, we have continued Gerard's tiltmeter programme and the records taken at Wellington have been analysed up to November 1981. With the much longer run of data, it has been possible to re-examine the validity of the earlier analysis<sup>2</sup>. It now seems that neither of the previously reported precursors is likely to be significant, as they can be attributed to a nonlinear response to rainfall combining with the statistical techniques used.

For example, there was a period of very heavy rain, causing local flooding, in

primitive precursors. There is therefore no positive indication that they originate from pluripotential cells with renewal capacity.

There is now a 20-year tradition of clonal analysis in experimental haematology. The basic approach is to ascribe potentials to precursor cells on the basis of the character of the clones they ultimately produce. We also subscribe to the converse, namely, not to attribute capacities that are not observed in the resulting clones. Blackett raises a legitimate question, namely, that the behaviour of colony-forming cells observed in the conditions of their assay could differ from their behaviour in steady-state marrow. This problem is inherent in the nature of colony assays whether performed *in vivo* or in culture. The way out will involve methods for specific and direct identification of 'stem' cells which have yet to be devised.

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December 1976. Much of the water ran off instead of soaking into the ground around the tiltmeter site, so the departure in tilt was not proportional to the total rainfall. When the data were corrected statistically for the effects of rain, this month was therefore over-corrected. The spurious signal thus generated was spread by the five months of lag correlation allowed. It was further shaped by the 24-month running mean, trailing the data points by four months, which was used to remove residual seasonal effects and trend. It then appeared to be a precursor to the magnitude 6.2 earthquake in January 1977.

We hesitate to express confidence in any of the results from this tiltmeter programme, as it has not been possible to correct adequately for rainfall. It seems essential to minimize the meteorological effects by site selection, and we suggest the use of bore holes several hundred metres deep from level terrain. Correlation with ground water level may then correct more appropriately for the effects of rain.

I thank colleagues at the Geophysics Division of DSIR, at Victoria University, and in this laboratory for many valuable discussions.

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# Machiavelli at the zoo

Robert Attenborough

**Chimpanzee Politics: Power and Sex Among Apes.**

By Frans de Waal. *Cape/Harper & Row: 1982. Pp.223. £8.95, \$15.86.*

IN CHALLENGING the idea that politics are unique to human society, Frans de Waal's book appears to come from a well-established mould. As with tool-making, art, the incest taboo, language, culture and so forth, the procedure is to draw on observational studies of a non-human species (chimpanzees here, as often before) and to interpret certain features of the animal's behaviour as representing at least the beginnings of what was supposed to be uniquely human: and the subject seems to invite renewed debate (exciting or wearisome, according to taste) between the radicals who like to see the arrogance of human uniqueness deflated and the reactionaries who prefer a more traditional view of the gap between man and animals.

Yet to plunge headlong into a set-piece debate would be to miss the point of this rather unusual book. The comparisons with human society actually take up only a small part of the book, most of which consists of a careful account of the social dynamics of a particular chimpanzee group. This group, a captive colony at Burgers' Zoo in Arnhem, has been studied intensively for some years by de Waal and others in "the strong Dutch ethological tradition" as de Waal rightly calls it: and what emerges from this study is fascinating in its own right. After a general introduction and character-sketches of the chimpanzees, de Waal deals in detail with some of the complexities of the group's social organization — two episodes in which one individual eventually replaced another as "leader"; the stabilizing processes of greeting, reconciliation and coalition; the relation of status to sexual activity; and other aspects of social life such as sex differences and food-sharing.

All this may sound interesting enough, but perhaps with little to distinguish it from a lot of primatology that has gone before it. There are, however, special things about de Waal's work. First and most straightforwardly, among chimpanzee studies it can claim to be, as Desmond Morris succinctly puts it in his foreword, "more natural than any other captive study, and more detailed than any wild study" — more natural because of the group's large open-air enclosure, more detailed because of the diffi-

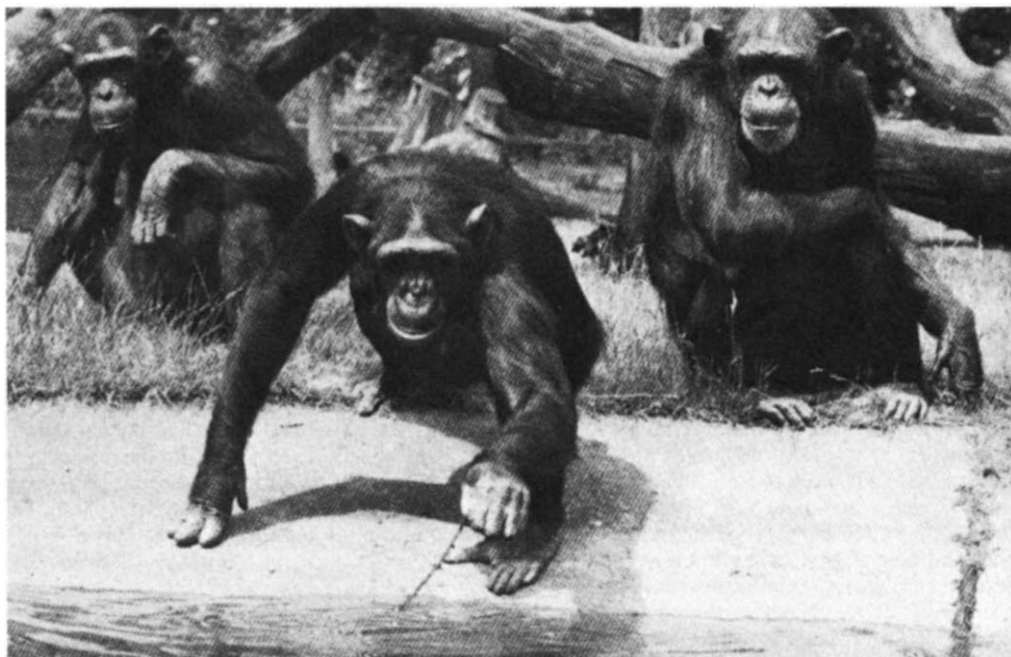
culties attending observations in the wild. This seems a fair assessment, despite the remarkable things achieved by wild studies.

The very complexity of the colony's patterns of social interaction and of its social history over a number of years is a second striking feature of the study. To some extent this complexity is of kinds that established ethological methods can document. But de Waal also goes into more problematic areas, raising the suggestions that, with their awareness of the changing network of relationships in the colony, individuals have policies towards their companions and make predictions about them, that their social actions are adjusted according to the support they expect from others, that they play off rivals against each other, that they form coalitions when they cannot win contests alone — in short, that their management of social status is planned in a rational, insightful and quite long-term way. These latter are no doubt what tempted de Waal to use the term "politics", and if truly present they are of course particularly interesting: but being sure of their presence is not easy, for reasons partly connected with the third quality for which the book is notable.

That third quality is the freedom with which de Waal attributes thoughts and feelings to the chimpanzees. He describes individuals as calculating, self-assured,

nonchalant or happy; he talks of respect, ambition, guile and resolution among them; and he uses phrases such as "playacting", "an atmosphere of conspiracy", "the excitement in their eyes". Descriptions of this kind, "anthropomorphism in its purest form" in de Waal's own words, run counter to the mainstream of modern ethology, which tries to avoid statements about the subjective experience of animals and the implied subjectivity of the observer. Several points can be made to suggest that this break with tradition is not as sharp as it seems: sometimes a subjective-sounding word is just the least cumbersome way of referring to an observed behaviour pattern; sometimes cautionary phrases such as "it seems as if . . ." are inserted; and sometimes de Waal is simply more relaxed about stating the kind of intuitive impression which ethologists often form but which they usually leave out of their publications.

Nonetheless, there remains an undeniably and unconventionally strong element of subjectivity in de Waal's approach. This is not done naively, or merely for the sake of readability (though readability was no doubt a consideration). Rather, in many instances it seems necessary for his purpose. If the proposition is that chimpanzees apply a kind of strategic intelligence to their social lives, how else could this be explored but by setting aside ethology's usual cautions and discussing what one thinks may be going on in chimpanzees' minds? Presumably the crux of the argument is that de Waal believes he sees behaviour which has no imaginable explanation except in complex mental processes of the kind he postulates. This is a difficult though not untenable position for an ethologist; indeed, others



Chimpanzee tool use at the Burgers' Zoo, Arnhem — "Amber has seen a piece of apple peel floating on the water and she is trying to reach it with a stick. Zwart (left) and Franje are curious to see whether she will succeed".

From *Chimpanzee Politics*

have arrived at a similar viewpoint in recent years. Personally, I have yet to be persuaded that ethology can dispense with objectivism, or that there are methods to study subjective phenomena objectively: but the field is an important and open one, and de Waal's view of it certainly deserves a careful hearing.

De Waal writes in everyday language, evidently with the general reader as well as the specialist in mind, but he manages to avoid many of the pitfalls of popular science writing. There are some lapses, however, both on particular subjects (e.g. social anthropology) and in coat-trailing to keep the reader's attention. A good impression of an ethologist's approach and evidence as well as his conclusions is given by such things as the inclusion of a few clear diagrams: I wish he had been bolder and supplied more of these, as well as cited evidence more specifically. Although I take with a pinch of salt the claim that "whole passages of Machiavelli seem to be directly applicable to chimpanzee behaviour", there is no doubting the fascinating complexity of chimpanzee social life: and de Waal's study of it is important both as empirical research and as a discussion of ideas. □

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## Why are there so few species of animals?

E. C. Pielou

### Resource Competition and Community Structure.

By David Tilman.

Princeton University Press: 1982. Pp.296.  
Hbk \$27.50, £23.70; pbk \$9.95, £8.70.

DAVID TILMAN has written an outstandingly good book. In it he develops a new, extremely well-argued approach to the perennially fascinating question: why have so many terrestrial plant species evolved (there are about 0.3 million) and how do they manage to coexist, in well-mingled species-rich communities, even though all have the same few needs of light, water, carbon dioxide and a few mineral nutrients? The profusion of plant species seems (until you read Tilman) far harder to account for than the profusion of animal species. Animals, even those at the bottom of the trophic pyramid, have available to them all the different parts of all plants, in round numbers about one million distinct resources. Tilman estimates that the number of species per resource is about  $10^4$  times as great in plants as in animals.

He shows how this high level of plant species diversity can be explained if one

postulates that the competitive success of a species depends on the relative rather than the absolute rates of supply of different resources. Spatial and temporal changes in resource ratios play an important role too. A hitherto unpublished figure (Fig. 35) gives isopleth maps of total nitrogen, extractable magnesium, and their ratio, in a  $12 \times 12$  m plot of soil. The intricacy of the small-scale pattern is noteworthy and might have been even more conspicuous if a three-dimensional study had been made, taking depth into account.

Tilman shows how niche diversification is possible among a collection of species even when they are competing for only two essential nutrients. His theory also explains very satisfactorily why species richness is nearly always greatest in habitats that are fairly poor in resources. However, the argument that temperate soils are often richer in nutrients than tropical soils, and that here at last is an explanation for the famous latitudinal diversity gradient, is unfortunate. As P. W. Richards, in his contribution to *Speciation in Tropical Environments* (Academic, 1969) has remarked, plant species diversity does not exhibit a clear latitudinal gradient (except in rain forest); the phenomenon is chiefly confined to animals.

Tilman develops his theory of competition for resources with a liberal use of isocline graphs. When the postulates are put on paper in this form, not only are the consequences that follow immediately visible but in addition the logical unfolding of his theory becomes a pleasure to follow.

The theoretical development is accompanied by careful and detailed discussion of a wide variety of actual communities ranging from cultures of diatoms and blue-green algae to forests. This brings me to what seems the only real weakness in the whole theory: no allowance is made for delays in the responses of a plant to changes in the rate of supply of its resources. Obviously blue-green algae respond faster than trees. The complete neglect of time lags is the one serious fault in the whole edifice; because the theory is so satisfying, I hope Tilman will be able to show that it is robust enough to survive the incorporation of this additional factor.

In a fascinating concluding chapter, Tilman sums up his answer to the question posed at the beginning of this review, which boils down to: why are there so few animal species? In a nutshell, his answer is that terrestrial plant species each require essential (non-substitutable) nutrients in specific ratios. Mobile animals, by contrast, tend to be able to substitute one resource for another if shortages occur. The contrast is thus between sessile specialists and mobile generalists, and the mystery disappears. □

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## Going to the pictures

P.W. Hawkes

### Digital Picture Processing, 2nd Edn.

By Azriel Rosenfeld and Avinash C. Kak.  
Academic: 1982. Vol.1 pp.434, \$35,  
£23.20; Vol.2 pp.343, \$29, £19.20.

THE FIRST edition of *Digital Picture Processing* appeared in 1976 and rapidly became the standard textbook of the subject, rivalled but not ousted by the few other good texts that appeared not long after. The subject is in almost explosive expansion however — at least two new journals and a "Progress" have recently been devoted to it — and the 1976 edition is no longer adequate as a course text.

The authors have added an immense amount of new material (Vol.1 alone of the new edition is as long as the 1976 book) and revised many passages that had become inadequate, though not to the point of desuetude. Specifically, whole chapters have been added on reconstruction from projections and on matching, most of which are new. The chapter on image compression, which badly needed bringing up to date, has been extensively rewritten so that the fast Karhunen-Loève transform and the cosine transform are now covered properly. It has also been possible to include block truncation compression.

The chapter on restoration has also been expanded, but is disappointing in one respect. The account of recursive methods in the first edition was by far the most readable I have seen of this difficult topic, and there have been many important developments since 1976; few of these have been incorporated though the section has been somewhat extended.

The other major theme of the work is picture analysis, which includes but transcends pattern recognition, and fills the second volume. The chapter on segmentation now includes a section on iterative relaxation methods for probabilistic segmentation, which will help the reader to understand fuzzy papers (in the technical sense), as well as new material on such topics as Hough transforms and edge detection. The final two chapters on representations and image description, notoriously difficult subjects for the beginner owing to the seemingly haphazard connections between different aspects, have been reorganized in a respectable attempt to impose a pattern on patterns.

I doubt whether it will be difficult to persuade users of the first edition to acquire the second. Those who are only just entering this fascinating but complex field will be very foolish not to equip themselves with this excellent, well-written and authoritative guide. □

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# Results from a volcanologist's laboratory

Michael F. Sheridan

**The 1980 Eruptions of Mount St. Helens, Washington.**

Edited by Peter W. Lipman and Donal R. Mullineaux.

US Government Printing Office/Microinfo, Alton, Hampshire: 1982.

Pp.844. \$35, £65.70.

THE 1980 activity of Mount St Helens is one of the great geological catastrophes to have affected the United States this century. Preliminary results and analyses of the monitoring by US Geological Survey scientists preceding, during and after the main crisis are documented in this massive book, Professional Paper 1250, which contains articles by more than 100 authors on topics relating to the eruptive phenomena and their effect on the surroundings.

It is certain that this eruption will have a long-lasting impact on volcanological research and hazard assessment throughout the world. As the main summary document released by the USGS, the book contains new data and interpretations of wide interest, ranging from basic geophysical observations to such topics as the use of fir tree leaves in estimating blast temperatures and the effects on the local blue-green algae. Each of the sections (volcanic events, geophysical monitoring, volcanic deposits, effects of the 1980 eruptions and analysis of potential hazards) includes a short summary of the principal research results on that topic.

Mount St Helens is a young volcano; most of the cone has grown within the past 2,500 years. The 1980 activity was similar to that documented for earlier events, consisting of ash-fall, pyroclastic-flow and lahar activity. Unexpected, however, was the great "directed blast" explosion of 18 May.

The main volcanic events included a series of steam-blast explosions beginning on 27 March, accompanied by the swelling of a large bulge on the northern flank of the cone. On 18 May an earthquake of magnitude 5 triggered failure of the oversteepened bulge that led to a gigantic rock-slide avalanche (2.3 km<sup>3</sup>) followed by the catastrophic "directed blast" and ending with a plinian eruption column of over 20 km that persisted for about 9 hours.

A wide array of geophysical experiments established prior to the cataclysmic event of 18 May, or shortly thereafter, tested various techniques for use in detecting precursor events. Seismic data provided the first indication that Mount St Helens might renew activity following a repose since 1857. After the phreatic activity in March, a high level of seismic activity coupled with growth of a huge bulge on the north flank of the mountain provided the strongest evidence that a major volcanic event could be expected. It should be noted that because no juvenile material had been erupted prior to the 18 May cataclysmic event, some volcanologists had argued that

no large eruption would occur and that a restricted zone was unwarranted.

Earthquake hypocentres preceding the main eruption defined a tight ellipsoidal pattern in a zone located less than 2.5 km directly below the bulge on the cone, supplying proof that the deformation was caused by the shallow injection of magma. Photogrammetric analysis defined the



Mount St Helens, May 20 1980. The volcano continues to erupt as mud-flows devastate the surrounding area.

shape of the bulge, the volume of which (0.11 km<sup>3</sup>) corresponded to that of the juvenile magma which was subsequently ejected. Although geodetic data recorded the remarkable northward migration (1.5 to 2.0 m/day) of the bulge, they failed to indicate a change in rate prior to the eruption.

Mount St Helens provided an exceptional opportunity for direct correlation of eruption phenomena with the resulting deposits. Photography, remote techniques and observations of deposits were made on a wide variety of volcanic events. In particular, the reconstructed time scale of the initial events of 18 May, based on Gary Rosenquist's photographs, provides exceptional first-order constraints of dynamic models for the avalanche, cloud development and "directed blast".

The "directed blast", the most important phenomenon, devastated an area of nearly 600 km<sup>2</sup> to the north of Mount St Helens and was generated during inception of the rock avalanche due to the release of pressure on the cryptodome and

its associated geothermal system beneath the bulge. However, the nature of this event — volcanic blast, pyroclastic surge or pyroclastic density flow — remains controversial. Further complicating the various interpretations given in the book, each of the authors involved uses a different terminology for similar phenomena and stratigraphic units. This is a pity since accurate analysis of the event is crucial for volcanic risk evaluation at other volcanoes with a record of hydrovolcanic activity.

Ash-fall deposits from all phases of the mountain's activity in 1980 provide some of the best data available for construction and testing of models of fall-out mechanisms. The eruptions before 18 May ejected only lithic and crystal fragments from the existing volcanic edifice, but the proximal fall-out deposits of the 18 May eruption are extremely complex in composition, with layers formed of materials from the "directed blast", vertical eruptive column, pyroclastic-flow ash clouds and fines settled from the atmosphere. As is shown in the book, distal ash-fall deposits from all of the magmatic eruptions are much simpler, showing a decrease in grain size and increase in vitric ash with distance.

Small but regular changes with time in the composition of the erupted magma (SiO<sub>2</sub> ranges from 64.4 to 60.3 per cent) indicate progressive tapping of deeper levels of a compositionally zoned chamber. The dark and light banded pumice in the 18 May deposit suggest mixing of magmas with slightly different compositions.

Also considered in the book are the far-reaching and long-term effects due to mud-flows and ash-fall deposits which caused extensive damage to river channels and civil works. However, toxic effects on algae were variable and reduction in water quality seems to have been short lived.

In retrospect, long-term hazard analysis based on prior geological mapping appears to have provided a good basis for interpretation of the short-term data gathered by volcano monitoring prior to the cataclysmic eruption. The major shortfall was the lack of anticipation of the size of the blast and associated surge, in part due to our poor understanding of hydrovolcanic phenomena and their deposits. Unfortunately, this phase of the eruption caused all of the deaths and most of the damage to property.

At Mount St Helens the ability to predict subsequent, smaller, events increased with time following the cataclysmic event, with four out of five minor eruptions being adequately predicted. Prediction of huge hydrovolcanic explosions at other volcanoes nonetheless still presents a great problem for volcanologists concerned with risk analysis. Analysis of the eruption of Mount St Helens, which this book documents so thoroughly, will prove to be a step forward. □

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# Glimpse of metabolic coordination

Helmut Sies

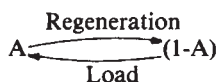
**Energy Metabolism of the Cell: A Theoretical Treatise.**

By J.G. Reich and E.E. Sel'kov.  
*Academic: 1982. Pp.345. £36, \$74.*

FOR those biological experimentalists sceptical of theorizing, Reich and Sel'kov make plain in the preface that their "... monograph does not present new facts. It is intended to explain a way of thinking". And, indeed, the authors are successful in conveying the message that you don't get more out of the model than you put in. At the same time, from reading the book it emerges that it is not only an intellectual challenge but also an aesthetic pleasure to attempt to unravel the strategies nature employs in governing the flow of energy and matter in biological systems.

The task the authors have set themselves — to describe, in theoretical terms, the energy metabolism of the cell — is monumental and also provocative; thermodynamic purists may ask whether there is any such thing that can be called energy metabolism, and experimentalists will always find that theory is too crude to account for the nuances found in the reactions they investigate. According to Reich and Sel'kov the way forward is to employ "specific methods of generalization" (whatever they may be) but never to completely lose touch with real data (whichever are at hand).

In a concise and well-written section, dynamics of flow in metabolic networks, steady states and their stability characteristics are presented. Homeostasis as an elementary phenomenon is described as a simple cycle of opposing fluxes:



One partner, (1-A), functions as an antenna to sense variations and to permit an effective rate control, while the other, A, is higher in concentration and less subject to fluctuation. (1-A), then, is the floating variable whereas A is the homeostatic variable. This theme is found throughout cellular metabolism and, accordingly, recurs in the book to explain adaptation as an elementary phenomenon as well as coupling, amplification and autocatalysis.

Triggers and oscillations permit dynamic responses in metabolism which may be considered more useful than monotonous responses, just as a zig-zagging rabbit is better off when hunted than one that insists on running in a straight line. Such is the value of multistationary states and hysteric effects, a topic developed in a remarkably clear fashion. In this context, probing of the dynamic structure of the so-called

futile cycles might attract research interest.

The authors (or their translators) evidently took much pleasure in gearing the book for English-speaking readers. In a section on time hierarchies, they explain that rapid components of motion approach their quasi-stationary state long "before you can say Jack Robinson" and, when calculating time elapsed, they take a train from London to Sheffield. In this section on time scales, however, the authors should have emphasized experimentation. Regarding relaxation kinetics, they feel that perturbation (excitation) experiments are elegant but expensive, and therefore look for simpler devices. This reasoning is hard to grasp, and in several similar instances the reader must accept flat statements unsupported by adequate data.

This very contractedness of presentation nonetheless leads to an excellent shorthand description of the group-transfer networks of near-equilibrium reactions. The passage on the occupancy and capacity of a near-equilibrium system serving as the "grey eminence" — for example in the protonation equilibrium or in the NADH/NAD<sup>+</sup> system — is superb. So is the definition of a *buffer* as a near-equilibrium system where every partner is in the same state of titration, as opposed to that of a *battery* as a near-equilibrium system maintaining an important metabolite in homeostasis; an example of the latter is the well-known ATP/creatine phosphate (phosphagen) system. Similarly, the logic behind

the concepts of "charge" (adenylate energy charge, phosphate energy charge and so on) is usefully exposed; one might even speak of a redox charge.

Having dealt with concepts, the authors go on to attempt an integrated overview of metabolism. Here their clear perception is inevitably blurred by the lack of fully satisfactory data, but their selection of experimental results is amazingly up-to-date and centres on those useful for consideration of steady-state conditions. The overview discusses building blocks (subsystems) as well as the special conditions to which the network may be exposed, including starvation, energy stress, storage and growth. In their discussion of Monod's growth formula and the Mitchell and Krebs-Kornberg reactors, however, one misses an explicit link to the Prigoginian school of thought.

Reich and Sel'kov have succeeded fairly well in heeding the dictum "Make everything as simple as possible, but not simpler". The book is a good compendium of modern metabolic concepts. But at least one thing is made not fully clear; at the end of the preface the authors promise to their wives to become available, after having written this book, "for more convincing and entertaining activities". What, one wonders, may those have been? □

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## Powerful description

D.O. Hall

**Dictionary of Energy.**

General editor Malcolm Slessor.  
*Macmillan, London/Schocken, New York: 1983. Pp.300. £25, \$29.95.*

WHEN (if?) the next energy crisis occurs we will certainly be much better prepared — if the wealth of information now available is anything to go by, as compared to the dark ages before 1973. However, with all this published work readily accessible, one of the problems is understanding the terms used by economists, biologists, physicists, engineers, politicians and leader writers who try to influence and educate us.

This excellent book claims to be

the most comprehensive energy dictionary ever produced. . . . The first part of each definition is intended to be elementary. Often a second part goes into more detail. Sometimes a diagram is inserted to assist understanding. . . . [It] aims to provide information for intelligent people searching for concepts, ideas, definitions and explanations in areas outside that of their own expertise.

Such a lengthy quotation is justified since I do not think I could summarize the objectives of this "interdisciplinary dictionary of energy" more succinctly.

The 12 or more pages devoted to nuclear energy just about make the book essential briefcase or knapsack stuffing for anyone concerned with the current nuclear debate — especially since the lucid description of nuclear reactors is so well complemented by all the other information on fossil and renewable energies. Economic terms and concepts are liberally "discussed" and defined so as to be understandable as they relate to the energy field. This comment also applies to definitions of energy accounting and ratios — this is such a muddled field at present that one can nearly justify any energy technology on efficiency grounds, solely by defining or ignoring the boundary conditions.

Included are a list of acronyms and abbreviations, a table of conversion factors (will we ever talk the same energy language?), and some useful addresses of national and international agencies. Of course, no dictionary is perfect. I couldn't find anything on Jerusalem artichoke or sunflower oil which are now so favoured as biomass sources of alcohols and diesel fuels. However, this type of complaint is common to all Scrabble players who vainly search such dictionaries in pursuit of high point-scoring words! □

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